

EDITORIAL

In accordance with the need for retrenchment disclosed by Doctor Greenman's letter (The Wistar Institute News of August 31st), the Editorial Board of The Anatomical Record is exerting every effort to cooperate with the publishers in reducing unnecessary expenses, and it welcomes the necessity for closer scrutiny of articles as an opportunity for improving the quality of the journal. It wishes to assure its contributors, however, that articles of excellence, suitable for publication in this journal, will continue to receive prompt publication.

As one of the organs of the American Association of Anatomists, The Anatomical Record gives preference to the kind of work being carried on by, and of special interest to, American anatomists, including border-line fields in the experimental sciences, but excluding neurology which, by agreement among Wistar editors, becomes exclusively the territory of The Journal of Comparative Neurology.

While, in general, it is desired not to accept articles exceeding twenty-five printed pages, the Editorial Board feels very strongly that the quality of an original contribution is not measured by its length. In the matter of illustrations, it holds the view that ability to display morphological structures pictorially, with distinction, is an essential qualification, and that good style and terseness in writing enhance the value of a scientific paper. With these objectives before it, the Editorial Board seeks the renewed cooperation of all contributors who are interested in advancing and maintaining the standards of American anatomical science.

EDWARD A. BOYDEN,
Managing Editor.

THE COURSE OF THE BLOOD THROUGH THE HEART OF THE CHICK EMBRYO DURING LATE EMBRYONIC LIFE

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ONE FIGURE

INTRODUCTION

There has long been a question as to the exact course of the blood through the heart of the chick embryo during the last week of incubation. Descriptions of the embryonic circulation through the heart at this stage are generally based on the morphological relations rather than on direct observation of the circulating blood in the living embryo.

Except for its smaller size, the heart of the fifteen-to-sixteen-day chick is externally very much like the heart of the adult bird. Blood enters the right auricle through the two anterior venae cavae from the anterior regions of the body and through the single posterior vena cava from the posterior regions of the body and its embryonic membranes. The pulmonary veins empty into the left auricle, but since they carry very little blood at this stage, they are relatively unimportant.

Internally, the heart of a fifteen-to-sixteen-day chick embryo resembles the adult bird heart, except for the presence of several foramina in the interatrial septum, i.e., the heart consists of four chambers, two ventricles separated by the complete interventricular septum, and two auricles separated by the perforated interatrial septum.

The blood may follow several possible courses through the heart during this period of development: 1) the caval streams

may pass through the right auricle with very little mixing, i.e., the blood from the anterior venae cavae entering the right auricle and passing through the atrioventricular canal into the right ventricle, and the blood from the posterior vena cava entering the right auricle and passing through the foramina in the interatrial septum into the left auricle and thence to the left ventricle. 2) The anterior and posterior caval streams may mix equally in the right auricle, so that each contributes equally to the blood content of each ventricle. 3) There may be an unequal mixing of the anterior and posterior caval streams in the right auricle so that the venae cavae contribute unequally to the blood content of each ventricle.

Lillie ('27) believes that the blood does not mix to a very great extent in the right auricle:

It is an interesting question to what extent the different kinds of blood received by the right auricle remain separate and receive separate distribution through the body. The blood poured in by the anterior venae cavae is purely venous, and it seems probable from the arrangement of the sinus valves that it passes into the ventricle of the same side, and so into the pulmonary arch and through the ductus Botalli into the dorsal aorta, and thus in part at least to the allantois where it is oxygenated. The blood coming in through the posterior vena cava is purified and rich in nutrition. . . . This blood appears to be diverted through the foramen of the septum atriorum into the left auricle, and thence to the left ventricle, and so out into the carotids and aortic arch. It would seem, therefore, to be reasonably certain that the carotids receive the purest and most nutritious blood. . . .

Patten ('27) does not consider the later development in regard to the circulation through the heart at all.

Wieman ('30) says:

There is in all probability a mixture of the two kinds of blood in the right atrium of which part passes directly into the right ventricle and part through the foramina in the interatrial septum into the left atrium and thence to the left ventricle.

What seems to be the best and most complete work along this line was done by Kellogg ('28) on mammals. In most of

his work he made use of living pig fetuses and a few dog fetuses. Kellogg's experiments consisted in injecting suspensions into the superior and inferior venae cavae and then observing the visible effects on the two ventricles and also by making controlled computations of the blood-suspension mixture. His results show, without much doubt, that the two caval streams do mix equally in the right auricle.

Bremer ('32), who has determined the path of the two vitelline streams in the heart of the forty-eight-hour chick embryo, finds that "the two streams might remain as separate entities, perhaps because of the colloidal nature of the blood, instead of coalescing as would streams of water. . . ." He found that the left vitelline stream entering at a lower level than the right, follows a spiral course around the right stream. "In this spiral course the left stream would pass first ventral to the right stream, then successively to the right of it, dorsal to it, and finally on its left side, the one making a complete turn about the other."

The evidence in this paper may be considered under two headings, viz., the results of injection experiments and anatomical findings.

INJECTION EXPERIMENTS

Materials and methods

In the experiments described here, fourteen-to-fifteen-day chick embryos were used. The shell was opened, the extra-embryonic membranes were carefully removed and the embryo was placed in a shallow dish containing 0.9 per cent saline solution. An incision was made on the ventral side in the pectoral region, exposing the heart and the vicinity immediately anterior or posterior to it, depending upon where the injection was to be made. This operation involved very little hemorrhage.

Injections were made after the method of Kellogg by means of a hypodermic syringe to which was attached a small glass tube drawn out into a capillary point. Such a glass needle is well adapted to this type of work, because any length or

shape of needle desired can be made. As all injections were made with the aid of a binocular microscope, the rate of passage of the suspensions could be observed through the walls of the glass needle. Starch (10 per cent) suspensions, India ink, and a blue water-soluble dye were used as injection fluids.

Blood was taken from the heart by means of identical glass needles, one attached to each arm of a Y tube by means of rubber tubing. The sharp ends of the needles were placed in the two ventricles and samples of blood were removed simultaneously from each ventricle by applying suction to the stem of the Y tube.

In the first experiment a suspension of India ink was injected into the right anterior vena cava, and changes in the heart coloration were noted. These changes were observed in both the auricles and ventricles. The coloration in the region of the ventricular apices, however, is considered most accurate, since in this region the ventricular walls are more nearly equal in thickness. Both auricles and both ventricles turned equally dark, the darkest portion being the apices of the ventricles. After a few experiments, the ink injections were discontinued as the ink seemed to have a toxic effect on the heart. Following injection the rate of beat was immediately reduced and soon ceased.

In the second experiment a water solution of Berliner Blau dye was used instead of ink. This solution seemed to have no toxic effect on the heart. The bright blue color was more easily seen through the walls of the heart than was the ink. For these reasons the dye was used in several experiments.

In all other injection experiments a 10 per cent suspension of starch was introduced into the selected vessel, usually one of the venae cavae, and in a few cases into the right jugular vein. As soon as possible, usually in less than twelve seconds, the blood was drawn simultaneously from each ventricle by means of identical needles described above. This fluid either had no effect on the rate of heart beat or accelerated it slightly, possibly because of the pressure introduced

by the syringe. In removing samples from the heart, only a very small amount of blood was obtained in any case. Such samples were immediately placed in separate glass tubes of small and equal diameter, to which had previously been added a given amount of potassium oxalate to prevent clotting. Because of their high specific gravity, the starch granules settled out first and occupied the lowest stratum of the column; next came a narrow stratum made up of the cellular elements of the blood and last the clear liquid. By such a column, a gross comparison could be made of the amounts of starch removed from each ventricle.

Starch counts similar to those made by Kellogg were attempted, but because of the relatively large size of the granules and the very small amount of blood obtained, accurate counts could not be made.

In the next experiment a slightly different technique was used. The embryo was opened as before, and in one case the anterior venae cavae were tightly clamped off with small clamps while the posterior vena cava was left undisturbed. Samples of blood were taken from each ventricle in the same manner as in the injection experiments, and the samples were placed in the same type of glass tubes and the height of the columns of blood were compared. Also the posterior vena cava was clamped off, allowing the anterior venae cavae to remain undisturbed and samples of blood from each ventricle obtained and placed in glass tubes. In all cases a given amount of potassium oxalate was previously placed in the tubes.

Results

The ink and dye experiments show, superficially at least, that the two ventricles receive the same amount of blood from each vena cava, since the coloration by these substances was similar in both ventricles. If the blood from the venae cavae does not mix equally in the right auricle, then the coloration by the ink and dye should appear deeper in one ventricle than in the other when the colored substances are injected into the vena cava. Since, as we find, the color

change is approximately the same in both ventricles, we may conclude that the blood from the venae cavae does mix to a considerable extent in the right auricle.

The experiments with the starch suspensions led to the same conclusions. Injections of starch suspension resulted in equal blanching of both ventricles in every case. As a rule the amounts of starch obtained by suction from the two ventricles were approximately equal. There were slight variations, but as these favored neither one nor the other ventricle, such variations were probably due to faulty technique, such as variations in the pressure used in injections. However, the pressure, as already mentioned, was rather carefully regulated by watching the progress of the starch granules through the glass needle.

The samples of blood drawn simultaneously from the two ventricles after clamping off the anterior or posterior venae cavae were in all cases approximately equal. If, as has been thought in the past, in the case of both birds and mammals, the streams from the venae cavae do not mix equally in the right auricle, the posterior caval stream proceeding to the left auricle and thence to the left ventricle and the anterior going directly to the right ventricle from the right auricle, then the clamping off of either the anterior or posterior should leave one of the ventricles practically empty for a short time. The fact that both of the ventricles contained the same amount of blood, in spite of the clamping off of either the anterior or posterior source, seems to show that both the anterior and posterior vessels contribute equally to the contents of both ventricles.

These three types of experiments, so far as they go, agree in indicating that the blood from the anterior and posterior venae cavae mixes equally in the right auricle and a mixture of blood from both sources reaches both ventricles.

The following criticisms of the experiments may be offered. In the first place, too few experiments have been completed to warrant the drawing of a definite conclusion. Also, abnormal conditions were introduced in opening the shell and re-

moving the embryo from its membranes. The slight hemorrhage always accompanying the opening of the body cavity, and the puncturing of an important vessel in the case of the injection experiments may introduce certain complications. Then, too, the introduction of the suction needles into the heart itself may have some modifying effect upon the results, due to the fact that pressure is exerted upon the heart and tends to interfere with its normal action and position. The ink and dye injections were advantageous as far as this last point is concerned since the progress of the ink and dye may be watched without disturbing the heart itself.

As Kellogg suggests, errors might be expected to result from the following factors:

1. The negative pressure exerted upon the heart in removing samples from the ventricles may result in abnormal circulation through the heart.

2. Introduction of a foreign substance into the blood stream may modify the results.

3. Injection of the starch suspension at a greater rate than the speed of the flow of the blood may result in an abnormal mixture of the blood in the heart.

As far as possible, precautions have been taken in performing these experiments, to minimize these factors.

ANATOMICAL EVIDENCE

Approximately fifty hearts (fifteen days) were dissected to see if the internal anatomy would corroborate the results of the injection experiments. As already stated, the internal anatomy of a fifteen-day chick heart is essentially like that of an adult bird. All the structures of the adult are present, although in a somewhat underdeveloped state. As the work was concerned with the course of the blood in the right auricle, these features were studied with the greatest care.

The right auricle is composed of two indistinct chambers which are in open communication with each other. These are the auricle proper and the sinus. The three large venae cavae open into the sinus which bears the large valves of the

right auricle. The right auricle communicates with the right ventricle through the atrioventricular orifice and with the left auricle through the foramina in the interauricular septum.

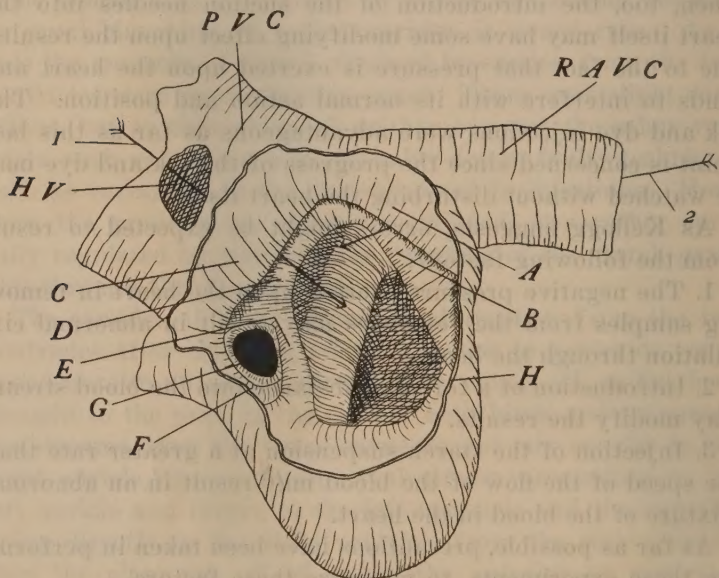


Fig. 1 A ventrolateral view of the right auricle with the right auricular wall removed. *A* and *B*, right and left valves of the right anterior vena cava; *C* and *D*, right and left valves of the posterior vena cava; *E*, ridge bounding anterior edge of left anterior vena cava opening; *F*, ridge bounding posterior and ventral edges of left anterior vena cava opening; *G*, opening of left anterior vena cava into the right auricle; *H*, cut edge of right auricular wall; *HV*, hepatic vein cut off at opening into posterior vena cava. *R.A.V.C.*, right anterior vena cava; *P.V.C.*, posterior vena cava. Arrows 1 and 2 show openings of right anterior vena cava and posterior vena cava into auricular chamber.

The posterior vena cava is a short thick vessel which opens into the dorsolateral region of the auricle (fig. 1, arrow 1). Its opening is guarded by two large parallel flap-like valves, the sinu-atrial valves (fig. 1, *C* and *D*). In the contracted state the mouth of the posterior vena cava is a long slit-like orifice that could easily be enlarged into a very large opening

by a small amount of pressure from the vein. Soft probes (feathers from embryos) were introduced into the posterior vena cava, but the angle at which the blood might flow from this vessel could not be determined with any degree of certainty. It would appear to be directed toward the anterior median ventral wall of the auricle if no other blood streams entering the right auricle were taken into consideration.

The right anterior vena cava opens into the sinus chamber of the right auricle at the anterior dorsal part just opposite the opening of the posterior vena cava (fig. 1, arrow 2). Its mouth is smaller than that of the posterior vena cava and is guarded by two large parallel valves (fig. 1, *A* and *B*) which are the continuation of the sinu-atrial valves mentioned in connection with the posterior vena cava. These valves are large flexible folds which arise from the posterior wall of the auricle ventral to the opening of the posterior vena cava and continue as semicircular folds to the anterior floor of the auricle. When the probable path of the right anterior vena cava stream is followed by the projection of a probe beyond the orifice, it would seem to be directed partly toward the mouth of the posterior vena cava and partly to a point ventral to it across the opening of the left anterior vena cava (fig. 1, *G*). Like the posterior vena cava, this hypothetical path described for the right anterior vena cava is the one which would probably be followed if no other blood stream were entering the right auricle.

The left anterior vena cava curves around the lateral side of the left auricle, courses across the heart behind the left auricle to open into the sinus portion of the right auricle. The opening lies in the posterior dorsal region of the auricle just ventral and slightly posterior to posterior vena cava opening (fig. 1, *G*). The opening of this vessel is smaller than the openings of the other venae cavae and is guarded by narrow ridges rather than by large definite valves found at the openings of the other vessels. One of these ridges (fig. 1, *F*) is the thick narrow continuation of the right sinu-atrial valves and bounds the posterior and ventral sides of the open-

ing, while a second short definite heavy ridge (fig. 1, *E*) bounds the anterior edge. This latter ridge is continuous at one end with the posterior end of the median or left sinu-atrial valve guarding the posterior vena cava and at the other end joins the posterior ventral part of the auricular wall. The hypothetical path followed by the left anterior vena cava stream would probably be across the cavity of the auricle toward the middle part of the curved lateral wall of the auricle and from there it might take various paths, but probably goes anteriorly and then medially again.

Three probable paths for the three main streams entering the right auricle have been mentioned. But these three paths have been based on the supposition that each stream was entering the auricular chamber as the main stream, and there is no direct experimental evidence in support of this supposition. However, if all three streams enter at the same time, as normally occurs, they are not likely to follow the separate paths outlined above, but are almost certain to be altered by each other and therefore mixed to some extent. In the case of the right anterior vena cava and the posterior vena cava whose openings into the auricle are practically opposite each other, a probe inserted in either will come out the cut end of the other with little or no displacement of parts. It is known, however, that the blood does not take this course. It can be seen by the arrangement of the surrounding structures that at least parts of these two large streams strike each other at some angle with a resulting mingling of the two streams. From the shape of the openings of these two venae cavae it is probable that a cross section of the streams from these two vessels would be more or less elliptical. As the auricular chamber is comparatively full of moving blood and since the left anterior vena cava stream appears to be directed toward the lateral wall of the right auricle, it would seem even more improbable that this stream could leave the right auricle without being mixed with other streams.

In no case is there a direct path from the mouth of a vessel to any opening leading either to the left auricle or the right

ventricle. The stream of the right anterior vena cava might be directed in the main toward the atrioventricular orifice, but even if unhampered by the posterior vena cava stream it would pass directly into the stream from the left anterior vena cava. The posterior vena cava seems to have the best possibility of passing through the right auricle in an unaltered course. It might be possible for at least part of this stream, which is the largest of the three, to be directed across the auricle ventral to the stream of the right anterior vena cava, toward the curved anterior wall of the auricle, and thence through the foramina of the interatrial septum. However, this is highly improbable, since two other large streams are flowing in the auricular chamber simultaneously. It seems almost impossible to explain, therefore, how any one or two of these three streams could enter and leave the right auricle without a thorough mixing with the others.

Although these results seem to be in direct contradiction to what might be expected from Bremer's work, cited in the introduction, there is really little basis for comparison between the two, since Bremer considered only the very young chick heart, while these experiments are concerned with the older stages only.

SUMMARY

1. Injection of India ink and a dye into the anterior and posterior caval streams results in an approximately equal discoloration of both ventricles.

2. The amounts of blood drawn simultaneously from the two ventricles after clamping off either the anterior or posterior venae cavae are approximately equal.

3. By injecting a starch suspension into the selected vessel and immediately obtaining samples of blood simultaneously from each ventricle, the amounts of starch were compared and found to be approximately equal.

4. The above points suggest that the blood from the anterior and posterior venae cavae mixes equally in the right auricle.

5. The anatomical evidence seems to bear out the experimental evidence.

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THE EFFECT OF THE MEDIA AND OF THE pH ON EMBRYONIC BRAIN CULTURES

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FOUR FIGURES

As is generally known, the embryonic nerve cells migrate and send out processes if they are explanted in lymph (Harrison, '07), in blood plasma (Burrows, '11), in certain salt solutions and Locke-Lewis solution (Lewis and Lewis, '12), in plasma and embryonic extract and in the liquor of the cerebrospinal fluid (Martinovec, '30). In addition to the growth of processes and the emigration of nerve cells, some cultures show the emigration of macrophages and the non-neuroblastic component of the ectodermic part of the brain (an epithelial-like glios membrane) but the emigration of the macrophages and of the ectodermal non-neuroblastic component takes place in semisolid media only (plasma and embryonic extract).

All the reactions of nerve cells in vitro described in the literature are concerned with the growth of processes, of plexus formation and the emigration of some cells. The reactions were always a further differentiation and never a multiplication.

In the following pages will be described the results of some experiments with different media by which it was possible to mobilize each neuroblastic cell in the explanted piece, and obtain a very quick further differentiation.

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Fig.1 Nerve processes from the mesencephalon of a seven-day chick embryo. Two days in vitro. Chicken plasma and embryonic extract. Formalin and acetic acid. Haematoxylin. $\times 100$.

Fig.2 Nerve processes and round cells from the mesencephalon of a seven-day chick embryo. Eighteen hours in vitro. Locke-Lewis solution. Living culture. $\times 100$.

SEMISOLID MEDIA

The nerve processes grow better in pure chicken plasma or in chicken plasma mixed with a small amount of weak embryonic extract than in a mixture of plasma and embryonic extract in equal quantities. In plasma and weak extract the processes are wavy and thin (fig. 1). The growth is slow; it takes two or three days before the processes are 1 to $1\frac{1}{2}$ mm. in length. There are no anastomoses between the processes, but bundles often form. On the second to the fourth day bulb-like swellings form in the processes. This is a sign of degeneration. Some nerve cells emigrate especially in liquefied areas; and after some days a great number of macrophages appear.

FLUID MEDIA

The Locke-Lewis solution is best for nerve cultures. It has several advantages compared with the plasma media; the nerve processes grow better, they are longer, they grow about three times as rapidly and the picture is clearer because the growth takes place on the cover glass. There is no growth of other tissues of the brain. It differs from the cultures explanted in plasma, inasmuch as the nerve processes often form rich plexuses.

The direction of growth of the processes during the first twenty-four hours is radial and they are about parallel with neighboring ones. On the next day, there is a tendency for the fibers to cross one another at right angles; this is especially true of the emigrated nerve cells and their processes.

The material which gave the best growth from the central nervous system of the chick was the mesencephalic part of the seven-day chick embryo.

Because the growing processes are better in Locke-Lewis solution than in plasma, experiments were made to obtain a better growth by changing of the components of the Locke-Lewis solution. First about one hundred cultures in Locke solution were made from the different parts of the central nervous system of chick embryos of different ages, but there

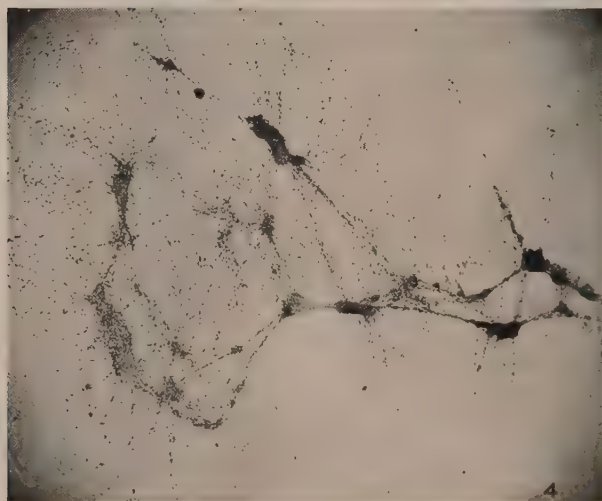


Fig. 3 Nerve processes and emigrated nerve cells from the mesencephalon of a seven-day chick embryo. Locke-Lewis solution without peptone and dextrose. Twelve hours in vitro. Living culture. About $\times 30$.

Fig. 4 Nerve processes and emigrated nerve cells from the mesencephalon of a seven-day chick embryo. Locke-Lewis solution without peptone and dextrose. Twenty-four hours in vitro. Living culture. About $\times 30$.

was no growth. Embryonic extract with Locke solution also gave no results. The Locke-Lewis solution differs from the Locke in that it contains chicken bouillon, peptone, and dextrose. The growth of the processes was the same in Locke-Lewis solution without peptone as with it. Cultures in Locke-Lewis solution without peptone and dextrose grow faster than in Locke-Lewis solution and the reaction of the explant is different because the wandering activity of the cells becomes very great (fig. 3). At the same time that the growth of the processes began, the emigration of the nerve cells also began. The explant separates into many very small pieces during the first twenty-four hours (fig. 4). The cause of this is the amoeboid movements of the ends of the nerve processes, which pull pieces of the explant in the direction of their growth. At the end of twenty-four hours, the whole explant is disseminated. Each nerve cell emigrates on the cover glass, and the culture is sometimes like a tissue spread. It is no longer possible to see the shape of the original explant. If the explant contained a bit of ependyma, this portion remained unchanged and isolated in its original place. The nerve cells are highly differentiated. They have one or two long, and often some short processes. Between the nerve cells and on the longer processes there are little round cells. We do not know what they are: they may be undifferentiated glioblastic cells or sheet cells or white blood cells. Anastomosis was never obtained between the nerve cells, but there were sometimes synapses: nerve processes have four to eight thin branches, and these sometimes wind around a nerve cell. Myelin never developed in vitro in our cultures.

Other procedures were tried in which different concentrations of the bouillon were used. Explantation in concentrations of 5 to 70 per cent, shows that the best concentration is 15 per cent, as it is in the Locke-Lewis solution. From this concentration down to 5 and up to 40 the growth of the processes becomes gradually weaker and weaker. When the concentration of the salts of the Locke solution was changed

growth took place in those solutions which were diluted with the same quantity of distilled water, but not in those in which the concentration was greater than one and a half times that of the Locke solution.

The last experiment was the testing and changing of the pH of the cultures. Clark buffers were used, because they are not toxic to the tissues (M. R. Lewis, '28). They are not toxic to the nerve cultures, as was shown by the following experiment.

The pH of the Locke-Lewis solution without dextrose or peptone, tested by the drop colorimetric method (Lewis and Felton), was found to be pH 6.8. Cultures were made with this solution and with another solution which contained 10 per cent of Clark buffer pH 6.8. There was no difference in the growth of the cultures made with the 10 per cent buffer, pH 6.8, or without buffer. Therefore, four sets of cultures were made with Locke-Lewis solution, without dextrose and peptone, buffered gradually from pH 5.4 up to pH 8.4. Twenty-four hours later, the best growth was in those solutions which were between pH 6.6 and pH 6.8. At a pH of 5.6 and 8.2 there was no growth.

The pH on the second set of cultures was tested at about twenty-four hours and in one set at about forty-eight hours. The results are shown on page 155.

From the table it is possible to conclude that the nerve cultures change the pH of the medium in the direction of pH 6.6 to 6.8. After the different pH solutions from 5 up to 9.8 were made with Clark buffers, they were kept in pyrex glass tubes, Arnoldized; and the next day the pH of the solutions was again tested. There was a change in the direction toward pH 6.8. The pH did not change again on the second day.

In order to compare the reaction of the nerve tissue with the connective tissue, four sets of cultures of the latter from seven-day chick embryos were made in Locke-Lewis without dextrose and peptone, but modified by Clark buffers so as to give solutions varying from pH 5.0 to pH 9.8. The best

growth was between pH 6.6 and 7.0. At a pH of 5.6 and 8.8 there was no growth. The connective-tissue cultures change the pH in the direction of pH 6.6 to 7.0. The results of these experiments confirm the observations of Lewis and Felton.

pH OF MEDIA	pH OF CULTURES		pH OF MEDIA	pH OF CULTURES	
	At 24 hours	At 48 hours		At 24 hours	At 48 hours
5.4	5.6	...	7.0	6.8	...
5.4	5.8	...	7.0	...	6.8
5.6	5.8	...	7.2	6.8	...
5.8	6.0	...	7.2	...	6.8
5.8	...	6.6	7.4	6.8	...
6.0	6.2	...	7.4	7.0	...
6.0	6.2	...	7.4	...	6.8
6.0	...	6.6	7.6	7.2	...
6.2	6.4	...	7.6	7.2	...
6.2	...	6.6	7.6	...	6.8
6.4	6.6	...	7.8	7.2	...
6.4	6.6	...	7.8	...	6.8
6.4	...	6.6	8.0	7.2	...
6.6	6.6	...	8.0	7.2	...
6.6	6.6	...	8.0	...	7.2
6.6	...	6.6	8.2	7.8	...
6.8	6.8	...	8.4	7.2	...
6.8	...	6.8			

I wish to express my thanks to Mrs. M. R. Lewis and Dr. W. H. Lewis for the assistance and suggestions they have contributed toward this study.

SUMMARY

Nerve processes grow better in Locke-Lewis solution than in semisolid media (plasma and embryonic extract). In Locke-Lewis solution, without peptone and dextrose, the nerve processes grow faster; and by emigration of the nerve cells, the explants became disintegrated. Dextrose retards this disintegration.

In nerve cultures formation of synapses sometimes takes place.

The pH-range of cultures in which nerve-cell emigration and growth of processes occurs is between pH 5.8–7.8. The optimum is pH 6.6–6.8. The culture tends to bring the solution to this pH, whether the original pH was higher or lower than pH 6.6–6.8. The pH properties of cultures of the connective tissue of the same chick embryos is nearly the same as for nerve cultures.

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MACROPHAGES IN CULTURES OF CHICK EMBRYO BRAIN

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TWO PLATES (EIGHT FIGURES)

Since the discoveries of Harrison ('07), many researches have been made concerning the growth of nerve processes in vitro. But the attention was directed to the nerve cells and processes, and only short notes were made to the effect that, in tissue cultures from the central nervous system there can be observed the emigration of mesenchyme cells (fibroblasts, wandering cells) and possibly glia cells. In the last two years, however, authors have paid more attention to the other kinds of cells of the nerve cultures.

Kapel found in 1929, in addition to the two kinds of emigrating nerve cells, a third kind of emigrating cell which he called the 'grob granuliertte runde Zellen.' They are characterized by many large granules; the whole cell body is full of them and the nucleus is pressed to the surface and sometimes is flattened. These cells are round and change their location by the ectoplasma processes. They are phagocytic and Kapel saw some amitosis among them, but no mitosis. He did not know from what these cells developed. He supposed they were glia cells, but he could not exclude the possibility that they were of mesenchymal origin, in which case they developed from clasmatoocytes, or from the few blood vessels present in the embryonic brain. He concludes that the connective tissue has not a great rôle to play, because after some changing of the medium brain-tissue cul-

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tures always give an epithelial and never a fibroblastic culture (Kapel, '27).

In 1930 and 1931, Costero reported the cultivation of the microglia cells in vitro from the brains of two-to-three-month-old human embryos, seven-to-twenty-day-old chick embryos, and the newborn guinea-pig. His attention was directed mostly to the granular wandering cells ('granulierte Wanderzellen'), which first appeared in the medium after twenty-four hours. They are large and round and have short, thin pseudopodium-like and thread-like processes, which always change length, size, and form. Their plasma is filled with large, strongly light-refracting granules. The nucleus is round or ovoid and often eccentric. He judges the vitality of these cells to be very great, because they move and live after the fibroblastic cells stop developing and moving in the same culture. Because of this fact, he could obtain pure microglia cultures by changing the medium twenty-four hours after the death of the fibroblasts. He concluded that these cells were microglia cells (Hortega cells), in spite of the fact that they look like cultivated monocytes and macrophages, because, 1) they changed their form, sent out pseudopodia, 2) wandered rapidly, and, 3) were phagocytic. These qualities are characteristic of the macrophages. Costero concluded that the microglia cells were mesodermal in origin because of their resemblance to cultivated monocytes and macrophages.

In the same year (1930), Wells and Carmichael published the results of their researches on the cultivation of the brain of chick embryos three to twenty-one days old. The authors found that the wandering cells of the brain cultures were nearly like the microglia cells and could be stained specifically by Hortega's impregnation. They could not get these cells from the retina, but from the brain only which contained blood vessels. They therefore concluded that these cells were mesodermal in origin. Wells and Carmichael certify to the mesodermic origin of these cells, too, by the fact that from the periosteum and from the limb buds of the chick embryos they also got cells of the same kind, and that they have the

same reaction with the vital staining. Their conclusion was that the microglia is probably a part of the reticulo-endothelial system.

In 1930, Verne distinguished three phases in the life of nerve-tissue cultures. The first was characterized by the growth of the nerve processes, the second by cell emigration, the third by growing of the nerve epithelium. He used material from six-to-ten-day-old chick embryos and rat embryos from the first part of pregnancy, because he thought that microglia could not be present in younger embryos. Verne writes that the cells which emigrate into the medium in the second phase of the nerve-tissue cultures belong to the neuroglia. He separated these cells into two types. In one type the cells are flat and polygonal with small granules of fat. These cells are grouped (two or three together). To the other type belong round cells which contain big neutral fat drops. This kind was found only after a few changes of the medium. The function of this type is to take up the parts of the dead neuroblasts ("Ils servent de véhicule assurant le transport hors de l'explant de substances en régression chimique"). He finds them similar to the wandering cells.

Verne considered the epithelium which appeared in the third phase as a representative of the glia, because he could show some glia fibrilles in it with the aid of Victoria blue and Mallory stain. According to him, the wandering cells (described in the second phase) come off from the epithelium membrane. Wandering cells and epithelium membrane are representative of glia. Therefore he concluded that the granular elements were of glia origin.

From all these data and from the comparison of the pictures in the publications, it is evident that Kapel's granulated, round cells, and Verne's large, wandering cells, rich in neutral fat drops, are identical with those which were described by Wells and Carmichael as similar to the microglia and by Costero as microglia. It is evident also that the authors differ as regards the origin of these cells. Verne considers them of ectodermal derivation; Kapel does not exclude the possibility of their mesodermal derivation, but he

rather favors their origin from epithelium; Costero, as well as Wells and Carmichael, are sure of their mesodermal origin.

I am convinced that these wandering cells which have been described in the tissue cultures of brain in the last three years are macrophages.²

THE DISTRIBUTION OF MACROPHAGES IN THE BRAIN

From the researches of Goldman ('09-'12) we know that macrophages can be found in most tissues, among these, the corpus striatum, the substantia nigra, and the retina. A similar distribution was reported by the researches of W. H. Lewis and Ch. C. McCoy ('22). Macrophages are in embryos as well (Beck, '22). From the publication of Beck we know that macrophages are in different parts of the seven-day-old chick embryo. He writes that the macrophages are in great numbers in those parts of the embryo where a loose mesenchymatic net is found, but in the brain (except the lobus opticus and the retina) macrophages are found in the absence of a mesenchymatic net.

MATERIAL AND TECHNIC

Hanging-drop cultures of the brain and of the spinal cord from chick embryos three to twenty days old and from young chicks one to two days old were made in Locke-Lewis solutions, in heparin plasma from adult chickens with a trace of embryo juice to permit coagulation, and in heparin plasma and embryo juice in equal quantity (mixed on the cover glass). Serial replantations of the explants in plasma media were also made every two to four days for varying lengths of time. More than 500 cultures were examined. Much care was taken to remove the pia mater from the brain or spinal cord in order that the cultures might contain only central nervous tissue. Living cultures were stained with 1:20,000

² The macrophages were discovered and described by Ranvier in 1890 to 1900. He called them clasmatoocytes. The name macrophage was used first by Metschnikow ('92). They are identical with the adventitia cells of Marchand ('99-'02); with the 'ruhende Wanderzellen' of Maximow ('02-'06); with the 'rhagiokrin' cells of Renault ('07), and with Kiyono's ('14) histiocytes.

diluted neutral red or with the same dilution of methylene blue. Fixed and stained cultures and sections were also made.

OBSERVATIONS

Fluid medium

Some hours after the explantation of the brain or spinal-cord tissues in fluid medium, the growth of nerve processes and the wandering of the neuroblastic cells began, both of these phenomena taking place on the cover slip (fig. 1). Near the edge of the piece a few macrophages were noticed. The growth of the processes continued for one to two days. The strongest growth was obtained in Locke-Lewis solution. This is the medium in which Lewis and Lewis ('12) had obtained the processes growing from the sympathetic cells of the intestine of chick embryos. In this some of the processes became 2 to $2\frac{1}{2}$ mm. in length. The number of macrophages did not increase during the life of the culture. There were never more than five to ten near the edge of the piece on the second day in one microscopic field. On the fifth or seventh day all cultures in the fluid medium were dead regardless of whether or not the medium was changed. I found no difference in these results if I took the material from the central nervous system of young or old embryos or if the material was taken from different parts of the same central nervous system; only the form of the emigrated nerve cells was a little different.

Plasma media

The cultures in plasma were somewhat different from those in fluid media. The growth of the nerve processes began a little later, the number of the fibers was greater during the first two days than in the culture with fluid media. According to the description of Olivo, the growth of the processes from the brain of the young embryos was intense only after one or two changes of the medium and the growth continued during more changes than in those cultures which were made from the brain of older embryos. In the latter the more

intensive growth was in the first days and passages, and the growth stopped earlier than in the cultures from young embryos.

Macrophages migrated near the edge of the piece some hours after explantation, but only in small numbers as in early cultures in fluid media. In the fluid media the macrophages did not increase in number after one day, while in the solid media, after a latent period of a day or two, they migrated out from the piece in very great numbers (fig. 2). They began to migrate from the older embryos earlier than from the younger ones. Their forms were quite varied: in the outer zone of the medium they were compact and round (fig. 3), near the explant they were long and multipolar (figs. 4 and 5). Over liquefied areas of the medium they stuck to and spread out upon the cover glass (fig. 6), and when near together came in contact with one another to form a veil-like pseudomembrane (fig. 7).

Serial replantation in plasma media. Serial replantations of the explants made every two to four days in fresh media over a period of two to four weeks exhibited the three types of phases of outgrowth. The first phase, the nerve-process phase, was characterized by the predominance of the outgrowth of the nerve processes, and varying numbers of macrophages. This phase was encountered in the primary cultures and in the first one or two replantations. The second phase, the macrophage phase, began after one or two replantations. In this phase the macrophages greatly predominated, but nerve processes were usually present. Within five to ten hours the macrophages migrated out in great numbers. After five or six transplantations the migration of the macrophages and the growth of the processes stopped, and the third phase began. Verne called it the epithelial or, more strictly, the glia phase. In this phases some macrophages can be seen for a while. The predominating feature of this phase was the outgrowth of an epithelial-like membrane (fig. 8).

DISCUSSION

It is evident that the wandering cells in the cultures from the central nervous system of the chick (embryos and young animals) are identical with Costero's microglia cells, and with the wandering cells of the brain-tissue cultures of Veratti, of Kapel, of Wells and Carmichael, and of Verne. Whether these cells are microglia cells, as Costero believes, or not is problematical, because Verne cultivated them from the brain of chick embryos from the first half of the incubation period, in which early stage there are as yet no microglia cells and because I find them in the brain and in the brain cultures of three-day-old chick embryos in which there are no microglia cells, as Hortega says in his newest description ('32), p. 486: "The microglia arises from polyblasts or embryonic cells of the meninges during late stages of development and its penetration and dissemination throughout the framework of the nervous tissue continues for a few days after birth." But I am sure they are macrophages, because Wells and Carmichael cultivated the same cell type from the periosteum and the limb buds of the chick embryos; I explanted in the same culture brain and liver or brain and skin of the chick embryos and I could not find any differences in the cultivated and emigrated macrophages from those three different tissues.

Until more exact knowledge is obtained regarding the development of the microglia, any statements regarding the identity of microglia and macrophages are not justified.

If the microglia belongs to the reticulo-endothelial system and develops from mesenchyme strictly from the polyblastic cells of the pia mater, as Hortega and many other authors say, then macrophages are identical with the microglia, but the observations of Lewis and Lewis ('25, '26) show that macrophages can come from monocytes. If the microglia belongs to the glia, according to the opinion of Schaffer ('26) and many authors, and develops from the ectoderm (Metz and Spatz, '24; Urechia and Elekes, '26; Creutzfeldt and Metz, '26; Bergman, '26; Pruijs, '27; Jakob, '27; and Besta, '29),

then the microglia is quite another component of the brain than the macrophages.

Costero identified the wandering cells (macrophages) of the brain cultures with the microglia cells, and from this he confirmed Hortega's opinion that microglia develops from mesenchyme and belongs to the reticulo-endothelial system. In this I differ with him, because the identification of the microglia with the wandering cells of the cultures (macrophages) is possible only if it is sure that microglia develops from polyblastic cells, but this is not a fact, hence the identification of the microglia with the macrophages cannot be accepted as certifying to the mesenchymatic origin of the microglia.

In agreement with Verne, Costero, and Veratti, I identify these wandering cells as the Gluge cells (Körnchenzellen). The macrophages are also identical with Cajal's ('13) third element ('el tercer elemento'), which looks like the monocytes. Thus I agree with the opinion of Pollak and Schaffer ('26), who supposed that only Cajal's third element (the round cell or apolar glia) is able to change into wandering (glia) cells (Gluge cells), during the exo- and endogenetic diseases of the brain parenchyma.

The origin of the brain-cultivated macrophages can be mesenchymal only, similar to the other macrophages. The question is, in the central nervous system do they come from the blood vessels or from the white blood cells, or from the mesenchymal adventitia of the blood vessel?

In answer to this question, we have the following facts: In every chick brain, from the three-day-old chick embryo to the old chick, we can find macrophages. This can be readily confirmed by the following experiment. From any part of the brain take a piece of tissue 1 to 2 mm. in diameter, put it in a 1:20,000 diluted neutral-red-Locke solution, cut up into smaller pieces. After three to thirty minutes, they were mounted under pressure between slide and cover glass. The macrophages can always be found in such supravitality stained preparations as reported by Beek for the seven-day-old chick embryo and by Lewis and McCoy for the rat. One

to two hours later, more macrophages are to be seen in the edge of the tissues, probably due to formation of granules or vacuoles and farther penetration of neutral red. In tissue cultures the macrophages migrate out in greater numbers than were contained in the original brain piece.

From the above facts, I conclude that in the central nervous system there are macrophages *in vivo* which *in vitro* are able to emigrate into the medium. They probably multiply in the explanted piece. This conclusion is based on the emigration of the great numbers into the medium, after successive replantation of the explant.

We cannot exclude from the genesis of the blood vessel and its adventitia, nor the blood cells, especially the monocytes, as sources of origin of the cells, because the observations of Lewis and Lewis ('25) show that macrophages can come from monocytes. In the light of this, it may be that the monocytes are Cajal's third element of the glia, and if the Hortege cells are certainly identical with Cajal's third element, then I agree with Costero that these *in vitro* cultivated cells are microglia cells, and then I add that microglia cells are macrophages. But, if so, then microglia develops in the early embryonic stages.

I wish to take this opportunity to express my appreciation of the kind hospitality, the assistance, and the many helpful suggestions which have been so generously given me by Mrs. M. R. Lewis and Dr. W. H. Lewis throughout the progress of this investigation.

SUMMARY

1. The wandering cells which emigrate out from the brain cultures of the three-to-twenty-one-day-old embryos and one-to-two-day-old chicks are macrophages (histiocytes).

2. They are identical with the Gluge cells and with the third element of Cajal.

3. Macrophages are present in every chick brain from the three-day-old embryo to the adult animal.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Nerve processes and nerve plexuses. One-day culture from the mesencephalon of a five-day chick embryo. Locke-Lewis solution. Living culture. $\times 100$.

2 Emigration of the macrophages. Four-day in vitro from the mesencephalon of a fifteen-day chick embryo. Chicken plasma and chick embryonic extract. First replantation. Formalin and acetic acid. Haematoxylin. About $\times 35$.

3 Round macrophages from the outer zone of the media. Five-day in vitro from the mesencephalon of a five-day chick embryo. Chicken plasma and extract. Second replantation. Formalin and acetic acid. Haematoxylin. $\times 600$.

4 Emigration of the macrophages. Four-day in vitro from the mesencephalon of an eight-day chick embryo. Chicken plasma and extract. First replantation. Formalin and acetic acid. Haematoxylin. $\times 150$.

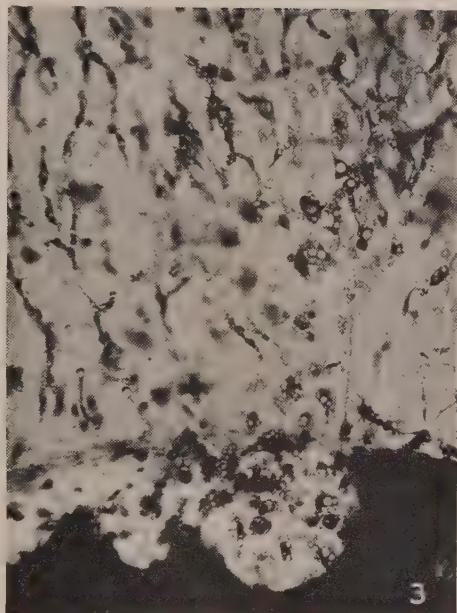


PLATE 2

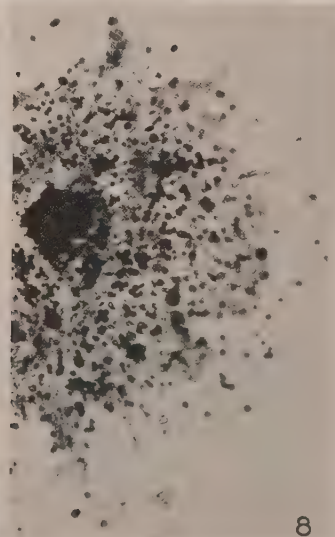
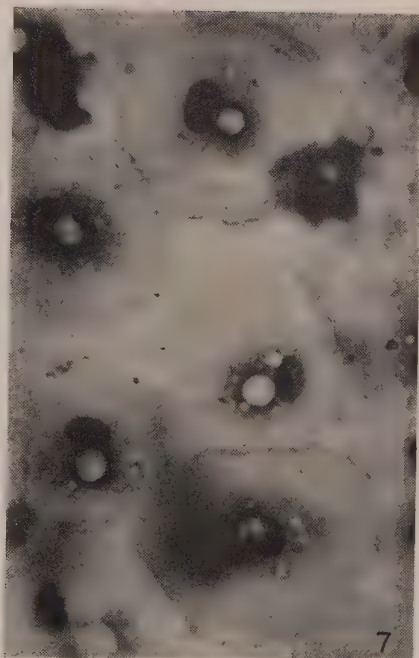
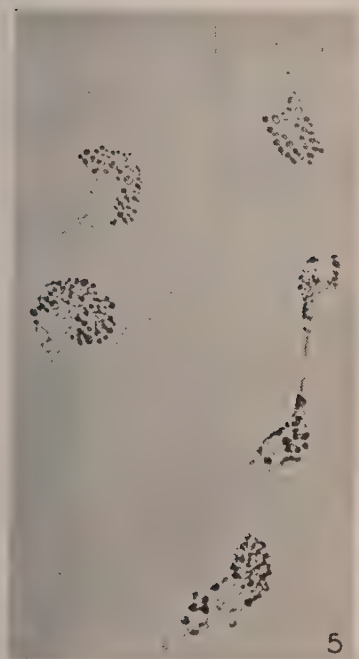
EXPLANATION OF FIGURES

5 Multipolar macrophages in the middle zone of the medium. Four-day in vitro from the mesencephalon of a five-day chick embryo. Chicken plasma and extract. First replantation. Living culture. $\times 660$.

6 Epithelial-cell-like macrophages. In the liquefied area of the medium. Seven-day in vitro from the mesencephalon of a twelve-day chick embryo. Chicken plasma and extract. Third replantation. Formalin and acetic acid. Haematoxylin. $\times 600$.

7 Pseudomembrane of the macrophages in the liquefied area of the medium. From the same culture as figure 6. $\times 600$.

8 Macrophages and epithelial-like membrane. Fourteen-day in vitro from the mesencephalon of a six-day chick embryo. Sixth replantation. Living culture. About $\times 50$.



PERFUSION OF THE INTERNAL EAR IN MAMMALS

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TWO FIGURES

INTRODUCTION

The circulation of the internal ear has been investigated from many points of view, but thus far no attempt has been made to study the effects produced by perfusing the perilymphatic space. Such a method is of interest in view of the recent work of Wittmaack ('19, '26, '28), Guild ('27), and de Burlet ('29). The experiments of Wittmaack have shown that changes are brought about in the cells of the organ of Corti by altering the chemical quality of the fluid in the perilymphatic space through the introduction of H and OH ions into the middle-ear cavity. Guild has brought forward clear experimental evidence that there exists normally what may be termed a circulation of the endolymph. He concludes that the endolymph is formed by the stria vascularis, and that it flows toward the basal end of the cochlear duct through the canalis reuniens into the sacculus, and from here into the ductus and saccus endolymphaticus. Also that it then leaves the membranous labyrinth by passing through the wall of the pars intermedia of the saccus to the numerous blood vessels of this region. Burlet has shown that these blood vessels traverse the connective-tissue trabeculae of the perilymphatic space on their way to the sensory end organs, and that there is a membrana limitans, formed by condensed connective tissue separating the pars superior of the labyrinth from the pars inferior.

The experiments here reported were planned and carried out with two objects in view: In the first place, to map out with different colloidal dyes the blood vessels and the perilymphatic space, respectively, of the same internal ear; in the second place, to determine what effect perfusion of the perilymphatic space would have upon the stria vascularis and upon the entire organ of Corti. Preliminary experiments in this direction were made on fishes at the Mount Desert Island Biological Laboratory, and on rabbits in the Department of Experimental Pathology at the Mayo Foundation. The final experiments which are now reported were carried out in the Pharmacological Laboratory der Rijks Universiteit Te Utrecht, Holland, with the generous cooperation and the stimulating encouragement of Prof. U. G. Bijlsma, the director, and Dr. A. P. de Kleyn.

MATERIAL AND METHOD

The apparatus (fig. 1) for perfusing the internal ear consists of two perfusion bottles (A) and (B), each immersed in a water bath regulated to a temperature of about 38°C. By means of two T-shaped glass tubes, each perfusion bottle is placed in communication with the metal cannula *C* (fig. 2) on the one hand, and with the water manometer *D* on the other. The compressed air passing into the bottle *E* supplies the necessary pressure to maintain a constant flow of fluid. It was found that satisfactory results were obtained when, with the cannula placed at the level of the ear of the cat, the tube *F* was lowered into the fluid until the water column in the distal limb of the manometer indicated a pressure of from 4 to 5 cm.

The solutions used in perfusing the perilymphatic space were of two kinds. First, the 1 per cent concentration of freshly prepared potassium ferrocyanide and iron ammonium citrate which Weed ('17) has shown to be isotonic with the body fluids; secondly, the graphite ink, 'Hydrokollag 300,' described by Drinker ('27), and prepared according to the modification of Higgins ('28). In later experiments, hyper-

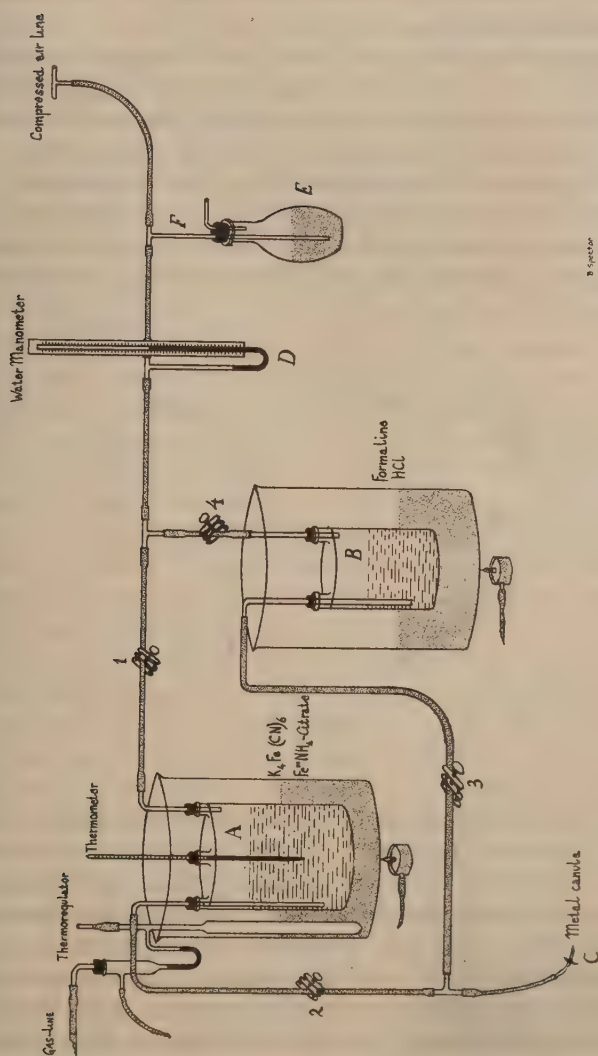


Figure 1

tonic and hypotonic solutions will be used for perfusing the perilymphatic space.

The operations were performed on living cats under deep ether narcosis. The animal was secured to the operating table, the trachea quickly exposed and cut, and a tracheal cannula inserted. The tracheal cannula was attached to a rotatory pump which maintained artificial respiration. The head was tilted to bring the left ear directly upward. The auricle and the cartilaginous portion of the external acoustic meatus were removed, and the inferior border of the temporal

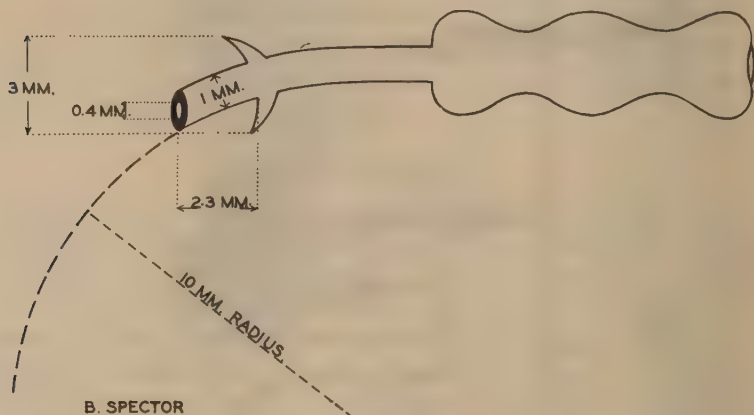


Figure 2

muscle was retracted until the ridge of bone which extends directly backward from the root of the zygomatic arch came plainly into view. When this bony ridge had been carefully chiseled away, the stapes and the stapedius muscle were exposed. The tympanic membrane with the malleus and incus and about 4 mm. of the mastoid process were then removed to give better access to the foramen ovale. By means of a periosteal elevator, the tip of the mastoid process and the inferior portion of the auditory bulla were dissected free, and projecting parts of the bone removed. This done, the septum which separates the auditory bulla into two unequal

chambers was cut away in order to expose the promontory, the dorsally placed fenestra vestibuli and the ventrally placed fenestra cochleae. The bleeding during the operation is moderate and easily controlled.

The perfusion apparatus was tested to see that no bubbles of air were present in any part of the system. With the aid of a binocular microscope, the stapes was removed and the cannula *C* inserted into the fenestra vestibuli, and secured by cotton swabs dipped in equal parts of vaselin and ox fat to prevent leakage. The fluid from reservoir *A* was then permitted to enter the scala vestibuli by opening the stopcocks 1 and 2, and closing 3 and 4. As the pressure in the perilymphatic space rose, the membrane closing the fenestra cochlea was punctured, so as to allow the fluid to escape freely. After two minutes of slow perfusion, stopcocks 1 and 2 were closed, and 3 and 4 opened so that the fluid from reservoir *B* flowed through the internal ear, resulting in the prompt precipitation of Prussian-blue granules. It is necessary at this point to emphasize the importance of this part of the technique. The tissues of the internal ear are fixed by this means without the need of removal, the perfusing fluid fixing the tissues. By varying the rate of flow from the cannula, it is quite easy to see that a circulation through both scalae is actually taking place.

Ten animals were operated on according to the technique described above. Seven of them were perfused with the Prussian-blue precipitating fluid, and three with hydrokollag ink. In the case of animals nos. 9 and 10, the entire blood-vascular system of the head was injected through the heart with hydrokollag, while the internal ear was perfused with the Prussian-blue fluid. The portion of the petrous bone containing the internal ear was removed from the skull, placed in Wittmaack's solution and sent to Prof. Dr. de Burlet, director of the anatomical institute in Gröningen, through whose kindness some of the preparations will be sectioned and others cleared by the method of Spalteholz. A report of the findings will be given at a later time. I am greatly in-

debted to Professor Burlet for his assistance in obtaining morphological orientation in this problem.

I desire to express my gratitude to Prof. H. D. Senior, of New York University, and to Doctor Mann, of the Mayo Foundation, for their friendly and helpful counsel during the preliminary stages of this work.

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THE INCIDENCE OF MIDDLE-EAR INFECTION IN THE WISTAR ALBINO AND THE LONG-EVANS HYBRID STRAIN OF THE NORWAY RAT¹

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The fact that middle-ear infections occur in rats on vitamin-deficient diets has been well established. Various investigators (Donaldson, '24; Nelson and Gowen, '30) have also reported its occurrence in rats otherwise apparently normal. In this study the incidence of middle-ear infection in otherwise healthy rats of two different strains is compared. Both these strains of rats are widely used for experimental purposes, so it is of importance to know whether one strain is more resistant to infections than the other.

MATERIALS AND METHODS

The original Wistar stock was obtained in 1927 from the experimental strain maintained at The Wistar Institute of Anatomy and Biology, Philadelphia. The Long-Evans strain is a result of a cross between several albino females and a wild gray male by Long and Evans of California over fifteen years ago (Long and Evans, '22).

The Long and Evans strain consists of rats having five coat colors, one of which is albino.

All of the animals of both strains are kept in the same room and given the same care. The diet which is abundantly supplied consists of: casein, 15 per cent, whole-milk powder, 10 per cent, sodium chloride, 0.8 per cent, calcium carbonate, 1.5

¹ Contribution from the Department of Anatomy, University of Minnesota, Minneapolis, and from the Department of Anatomy, University of Utah, Salt Lake City.

per cent, butter (unsalted), 5.2 per cent, and whole ground wheat, 67.5 per cent.

The stock animals are somewhat selected in that the litter number is always reduced to six at the time of birth. At weaning time (three weeks) all young less than 35 grams in weight are usually discarded.

Four hundred and sixty-two animals were completely autopsied, 222 of which were Wistar albinos and 240 of which were of the Long-Evans strain. Animals were autopsied at approximately three weeks, twelve weeks, and one year of age. They were quite equally divided as to sex (table 2). All animals were healthy, practically free from parasites, and apparently superior in every way (Freudenberger, '32).

TABLE 1
Incidence of middle-ear infection according to age

	NUMBER OF RATS	NUMBER WITH MIDDLE-EAR INFECTION	PERCENTAGE INCIDENCE
3 weeks	142	17	12
12 weeks	123	19	15
1 year	197	84	43
Entire group	462	120	26

The thin roof of the tympanic cavity was removed to note the presence or absence of pus.

OBSERVATIONS

According to age

One hundred and forty-two rats were examined at three weeks of age, of which number 17, or 12 per cent, showed exudate in one middle-ear cavity or both. One hundred and twenty-three were observed at twelve weeks, of which 19, or 15 per cent, showed the ear infection. Of 197 animals at one year of age, exudate was found in the middle-ear cavities in 84, or 43 per cent. Out of the total number of 462 rats (table 1), 120, or 26 per cent, had the middle-ear infection. These observations agree with those of Nelson and Gowen

('30) in that there is an increased susceptibility to the infection with increasing age. The animals are apparently about three times as susceptible at one year as at twelve weeks. However, susceptibility apparently increases very little from three weeks to twelve weeks of age.

According to sex

The tympanic cavities of 74 females three weeks of age were examined. Thirteen, or 18 per cent, showed pus in one or both cavities. Four, or 6 per cent, of the 68 males at three weeks showed middle-ear infection. Eight, or 14 per cent, of 57 females at twelve weeks of age had the ear disease, while 11, or 17 per cent, of 66 males at the same age showed it. At

TABLE 2
Incidence of middle-ear infection according to sex

	NUMBER OF RATS	NUMBER WITH MIDDLE-EAR INFECTION	PERCENTAGE INCIDENCE
3 weeks females	74	13	18
3 weeks males	68	4	6
12 weeks females	57	8	14
12 weeks males	66	11	17
Year-old females	99	35	35
Year-old males	98	49	50
All females	230	56	24
All males	232	64	28

one year of age, 35, or 35 per cent, of the 99 females showed the infection, while 49, or 50 per cent, of the 98 males had it. If the age groups are combined, 56, or 24 per cent, of 230 females studied had middle-ear infection, while 64, or 28 per cent, of 232 males had it. Thus, the infection was found to be slightly more prevalent in the males (all ages combined) than in the females. Considering the ages separately, it is more prevalent in the males at one year and at twelve weeks of age, but the females are more susceptible at three weeks. Nelson and Gowen ('30) found the incidence of infection to be greater among the males. Their animals were all more than three months of age, so the two sets of data agree as to this small sex difference.

According to strain

Of 60 Wistar animals examined at three weeks of age, 8, or 13 per cent, had middle-ear infection. Nine, or 11 per cent, of the Long-Evans rats examined at this age showed the infection. At twelve weeks of age 13, or 21 per cent, of 63 Wistar rats had exudate in the middle-ear cavities. It was found in 6, or 10 per cent, of the 60 Long-Evans animals examined at a corresponding age. Fifty-nine, or 60 per cent, of 99 Wistar animals one year of age had the infection, while 25, or 25 per cent, of 98 Long-Evans animals had it at one year. Thus, the Long-Evans strain of rats are apparently much more

TABLE 3
Incidence of middle-ear infection according to strain

	NUMBER OF RATS	NUMBER WITH MIDDLE-EAR INFECTION	PERCENTAGE INCIDENCE
3 weeks Wistar	60	8	13
3 weeks Long-Evans	82	9	11
12 weeks Wistar	63	13	21
12 weeks Long-Evans	60	6	10
Year-old Wistar	99	59	60
Year-old Long-Evans	98	25	25
All Wistar	222	80	36
All Long-Evans	240	40	17

resistant to this infection than the Wistar. The difference is quite marked except at three weeks. If the age groups are combined, 80, or 36 per cent, of 222 Wistar rats had middle-ear infection, while the infection was found in only 40, or 17 per cent, of the 240 Long-Evans rats examined.

Incidence in the albinos of the Long-Evans strain

Sixty-eight of the 240 Long-Evans rats examined were albinos. Of these 68, 11, or 16 per cent, showed middle-ear infection. It will be noted that this percentage is practically the same as that (17 per cent) found for the strain as a whole and that it is not similar to that (36 per cent) found for the Wistar strain. This is in agreement with Freudenberg's

observations ('32) on many other characteristics of the Long-Evans strain. He found that the albino Long-Evans rats were not essentially different from the pigmented ones.

DISCUSSION

The diet which these rats were fed is apparently adequate in every way, so the high incidence of middle-ear infection found is probably not due to a lack of vitamins.

Donaldson ('24) states that about 1 per cent of the rats in The Wistar Institute colony show middle-ear disease. However, in a recent personal communication, he states that he now finds the incidence to be much higher than that recorded in 1924.

Jackson ('30), in his work on the effects of high sugar diets, used rats of the Long-Evans strain and autopsied them when they were from five to seven months of age. He found middle-ear infection in 40 per cent of those rats on sugar diets as compared with 47 per cent in the starch-fed controls. The diets used may have caused the animals to be more susceptible than normally, since pus was found in the tympanic cavities in only 25 per cent of my Long-Evans rats at one year of age.

Nelson and Gowen ('30) report an incidence of middle-ear infection of 32 per cent in albino rats between three and four months of age and an incidence of 69 per cent in albino rats over one year of age. These figures are only slightly higher than mine for Wistar rats of corresponding ages. Their older group of rats had died from natural causes or were culled on account of failure to breed, so can hardly be considered normal. This fact may account at least partly for the higher incidence (69 per cent) of infection in this group.

In the animals which I examined the ear infection was bilateral in 57 of the 120 cases. The right ear was infected in 29 of the 63 animals with a unilateral lesion. The fact that in unilateral lesions one ear is about as likely to be infected as the other agrees with Nelson and Gowen's results ('30).

The fact that the rats of the Long-Evans hybrid strain are much less susceptible to middle-ear infection than those

of the Wistar strain may be another example of hybrid vigor. Freudenberger ('32) has pointed out that the Long-Evans rats are also apparently more resistant to the so-called rat pneumonia than the Wistar rats. However, these differences in resistance to infections in the two strains may be just strain differences, since the two strains differ in many ways (Freudenberger, '32). Nelson and Gowen ('30) found that a group of wild rats showed 1 per cent middle-ear infection. As they state, the low incidence in this group may be due to the more natural environmental conditions to which wild rats are subjected.

SUMMARY

Data are presented on the incidence of middle-ear disease in the Wistar albino and the Long-Evans hybrid strain of the Norway rat. The rats were all healthy and in good condition. The rats at one year of age were about three times as susceptible to middle-ear infection as at three weeks of age, but were only slightly more susceptible at twelve weeks than at three. The females are slightly more resistant than the males. The infection occurs much more frequently in the Wistar strain than in the Long-Evans. Exudate was found in the middle-ear cavities of the albino Long-Evans rats in the same percentage as found in the pigmented rats of this strain. The infection was bilateral in about one-half the cases. In the unilateral lesions it occurred with equal frequency in the right and left ears.

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CYTOPHYSIOLOGICAL STUDIES OF THE NEPHROCYTES OF UNISEGMENTAL AGLOMERULAR AND GLOMERULAR NEPHRONS

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THREE PLATES (ELEVEN FIGURES)

As a part of a general program of a study of the comparative physiology of the kidney being carried out in this laboratory, an attempt has been made to correlate the cytological and functional changes occurring in the nephrocyte or renal cell. This research has been undertaken on the very simple nephrons in marine teleosts in order to avoid the more complex structure occurring in higher forms. The tubule of the toadfish (*Opsanus tau*) was used as an example of an aglomerular nephron and the tubule of the sculpin (*Myoxocephalus octodecimspinosus*) as a glomerular nephron. The nephron of the toadfish is unisegmental and composed of only one type of epithelial cell; the nephron of the sculpin is morphologically unisegmental, but from a strictly cytological point of view is composed of two types of cells, different in the shape of the Golgi substance. The nephron of the sculpin was nevertheless compared with that of the toadfish, because no simpler glomerular nephron is known.

At any given moment, the individual nephrocytes are not all in the same physiological state. To correlate, therefore, the function of the kidney as a whole with that of its constituent cells, it is necessary to make a quantitative cytological study of a number of cells and apply statistical methods. Such a study is particularly difficult by the ordinary methods

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in which sections of tissue are used, because any such section is not representative of the organ as a whole and cells cannot be studied in their entirety. A technique was developed by which homogeneous aqueous cellular suspension could be obtained permitting study of the nuclei, the impregnated chromophil portion of the Golgi complex, the chondriome, and the apical border of the cell. By this technique one could obtain a cellular population from which could be calculated the median normal morphological values for a given functional moment. Careful controls were made to determine that with the method utilized there was no modification of the shape or structure of the cells as examined in fresh material. But a standard method by which at any functional moment it would be possible to have in every type of tissue sure and constant results is not yet reached, especially in osmic macerations, where the results are quite inconstant. Most of the conclusions here reported are for that reason based upon sectioned material. The functional state was controlled in each case where material was taken for cytological study.

Since the technical details and the numerical results will be reported later, only a brief summary of the general conclusions reached for the normal animal will be given here.

1. The nephrocytes, except for the shape of the Golgi substance, are fundamentally similar in glomerular and aglomerular nephrons and, with the above exception, do not differ from those of the first portion of the proximal convoluted segment of the mammalian tubule.

2. The nephrocyte of both nephrons possesses a long, flagellated diplosome extending into the lumen (figs. 3 and 10).

3. The nucleus has an irregular surface and is varied in shape and position.

4. The chondriome in the aglomerular nephron is irregularly arranged and spread throughout the cytoplasm. In the glomerular nephron, on the other hand, it is more similar to that of the mammal (figs. 3, 10, 11).

5. The substance 0 of the Golgi complex in the aglomerular nephron consists of voluminous, rather spherical masses, vari-

able in number, volume, and position, depending upon the functional condition of the cell (figs. 2, 4, 9). In the glomerular nephron, it may be either in the basal part of the cell (scattered, as rods) or in the supranuclear zone (as masses) (figs. 6, 8, 10, 11). These differences in the shape and position of the Golgi substance cannot be interpreted as functional changes, because cells with rods and cells with masses are regularly disposed in two different portions of the tubule: rods in the proximal, and masses in the distal.

6. The apical zone may assume a dome-shaped, straight, or cup-shape border. The so-called brush border or striated border is not constituted by isolated single filaments, but consists of striations in the cytoplasm.

7. The passage of substance from cell into tubular lumen is demonstrated by finding, in the apical part of the cell and in the lumen, of vesicles enclosed in a membrane staining, though not consistently, with osmic acid. This indicates actual secretion by the cells in the glomerular as well as the aglomerular nephron, because fixations were always made in isotonic solutions, and vesicles were also detected in supravital conditions by teasing tubules absolutely well preserved (fig. 1).

8. Nephrocytes in the same nephron present different cytological pictures depending on their state of activity.

9. The cells of the collecting segment manifest cytological changes in different functional states, thus indicating a possible participation in the elaboration of urine.

The ability of the kidney to increase its excretion of water offers one of the simplest methods of changing its functional state. A study was therefore made of the cytological changes accompanying diuresis. Magnesium sulphate produces diuresis in both the aglomerular and glomerular kidneys. The nephrons do not all respond simultaneously, due most likely to the different stages of activity of the cells. However, five or six hours after an injection of this substance, there is a great tendency toward simultaneous activity of all nephrocytes. As a result of stimulation by magnesium sulphate, there is marked diminution in the volume of the cells (particu-

larly the infranuclear zone), and marked rearrangement of the chondriome and chromophile substance of the Golgi complex. The latter is reduced with the formation of smaller masses than occur in the normal, diffused through the basal zone (figs. 2, 4, 9). After repeated injections, no further cytological response is obtained. The cytological changes noted above are quite similar to those encountered in the cells of such glands as the pancreas under the action of pilocarpine.

Urea, which differs from magnesium sulphate in not being highly concentrated by these kidneys and in producing less diuresis, produces very slight cytological changes as compared to those encountered with magnesium sulphate. There is a slight change in the shape of the chondriome and some diminution of the chromophil substance of the Golgi complex. It appears that urea does not exhaust the renal cell or modify the functional cycle of the nephrocyte.

Caffeine, which produces no diuresis in the toadfish, does not change the structure of the nephrocyte. This drug was not tried in the sculpin.

Hypotonic sodium chloride produces no diuresis in the aglomerular kidney, but does give a definite diuresis in the glomerular kidney. At this is a glomerular diuretic, no cellular changes are observed in the cells of either nephrons.

PLATES

PLATE 1

EXPLANATION OF FIGURES

1 Opsanus tau. Teased tubule in isotonic solution, stained with neutral red (Ehrlich). There are visible liposomes in the apical part of the cells, and transparent vesicles in the lumen. The picture was made when the cells were still alive: the nuclei are not stained by the neutral red and chondriome is not granular. The transparent vesicles indicate actual secretion.

2 Opsanus tau. Transverse section of a nephron with silver impregnation of the Golgi substance (DaFano method). $\times 1500$. Functional state: chloride-free. The Golgi substance consists of rather spherical masses, which seem to correspond to the liposomes stained by neutral red.

3 Opsanus tau. Transverse section of nephrons, fixed in isotonic formol-Regaud and stained with iron-hematoxylin. $\times 1500$. Functional state: chloride-free. A) Filamentous mitochondria irregularly scattered in the cytoplasm, which presents clear zones corresponding to the Golgi complex. Nuclei with different shapes; flagellated diplosome extending into the lumen; border of the cells homogeneous; secretion vesicles in the lumen. B) Narrow lumen. In the same tubule portions with large and narrow lumens are in irregular alternation.

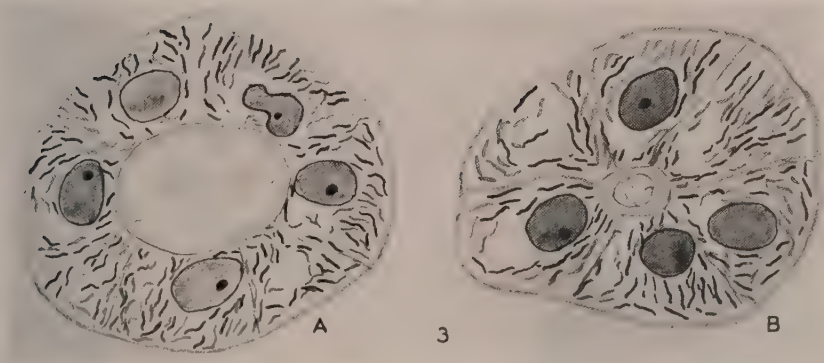
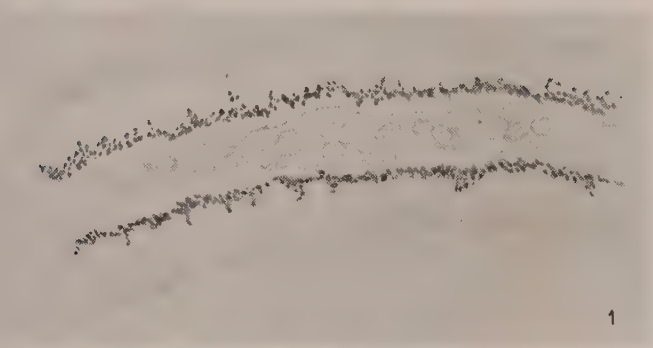


PLATE 2

EXPLANATION OF FIGURES

- 4 Opsanus tau. Transverse section of a nephron with silver impregnation of the Golgi substance (DaFano method). $\times 1500$. Functional state: diuresis by severe injuries to the skin. Very large lumen; remarkable reduction of the Golgi substance.
- 5 Opsanus tau. Transverse section of a collecting tubule, fixed in isotonic formol-Regaud and stained with iron-hematoxylin. $\times 1500$. Nuclei toward the basal part of the cells, filaments of the chondriome short and small, cuticular border, and flagellated diplosome. Connective tissue around the tubule.
- 6 Myoxocephalus octodecimspinosus. Transverse section of the distal part of the nephron. Silver impregnation of the Golgi substance (DaFano method). $\times 1500$. Functional state: chloride-free. Chromophil substance of the Golgi complex para and infranuclear in the shape of rods.
- 7 Opsanus tau. Transverse section of the preterminal portion of the nephron. Smaller amount of chondriome. Filaments shorter and smaller, some granules. Fixed and stained as figure 2. $\times 1500$. Functional state: chloride-free.
- 8 Myoxocephalus octodecimspinosus. Transverse section of the proximal part of the nephron. Silver impregnation of the Golgi substance (Da Fano method). $\times 1500$. Functional state: chloride-free. Chromophil substance of the Golgi substance in the apical part of the cell, and consisting of small, rather spherical masses.



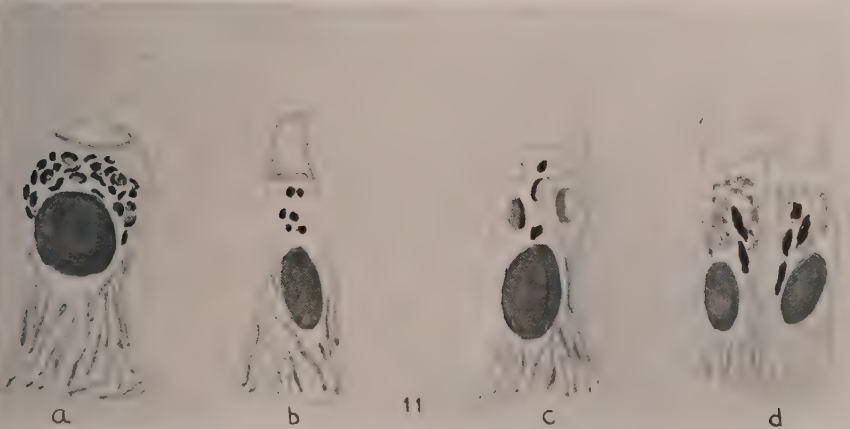
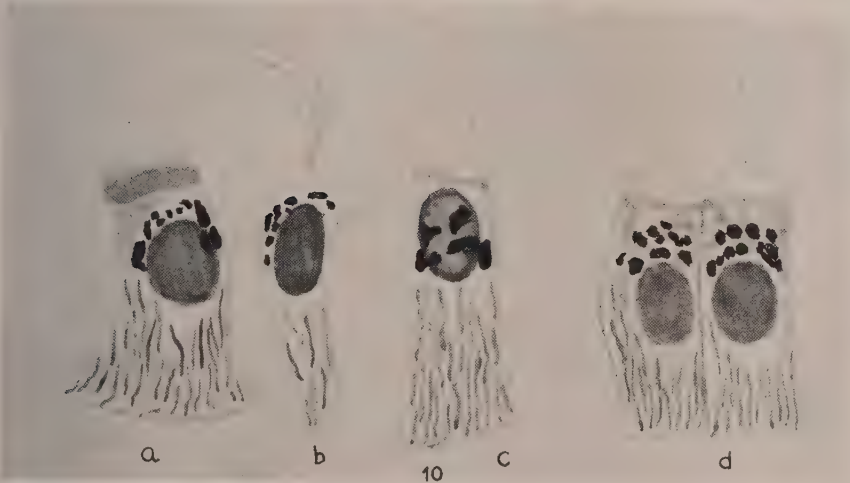
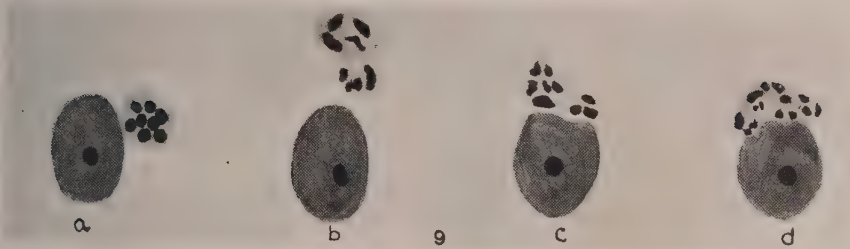
PLATE 3

EXPLANATION OF FIGURES

9 *Opsanus tau*. Golgi fields of normal isolated cells after osmic impregnation. In d., centrioles in the Golgi field? $\times 2700$.

10 *Myoxocephalus octodecimspinosus*. Normal isolated cells after osmic impregnation. $\times 2700$. Osmiophil portion of the Golgi complex, filaments of the chondriome, flagellated diplosome on the border. In b, a ciliated cell. In a, c, d, different shapes of the border.

11 *Myoxocephalus octodecimspinosus*. Normal isolated cells after osmic impregnation. $\times 2700$. In d, rod-shaped chromophil portion of the Golgi complex, in cells with cup-shape border.



A RARE INTESTINAL ANOMALY OF EMBRYONIC ORIGIN

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THREE TEXT FIGURES AND ONE PLATE

INTRODUCTION

Some years ago I reported in this journal (Papez, '21) a malposition of the duodenum observed in a subject in our laboratory. The case was compared with a number of similar ones reported in literature. The condition was interpreted as an incomplete rotation of the duodenum around the embryonic root of the mesentery. This embryonic root was designated the mesenteric pedicle to distinguish it from the mesenteric root of the adult which extends by adhesion to a considerably lower level than the original embryonic root around which this rotation of the intestines takes place.

It has been shown by Mall ('97) that the coils of the small intestines beyond the duodenum are extruded into the cavity of the umbilical cord in embryos up to 31 mm. in length. Jackson ('09) has shown that during this period the volume of the liver as compared with that of the body attains its maximum size. This spatial dominance of the liver and the extra-abdominal position of the small intestines is well shown by Mall's figures and by the Jackson models.

During this stage, the coils of the jejunum and ileum are developed within the cavity of the umbilical cord and constitute a normal umbilical hernia. The umbilical orifice remains narrow and through it the intestines are suddenly withdrawn in embryos from 28 to 32 mm. in length, as Mall has shown.

While the intestinal coils occupy the umbilical cavity, they are connected with the posterior abdominal wall by a long, narrow, and somewhat rounded mesentery which contains the mesenteric vessels and within the base of its pedicle the pancreas. On the right of this is the duodenum, on the left is the transverse colon, both extending toward the umbilical orifice to join the extruded parts of the small intestine. It is during this stage that the lower part of the duodenum travels around the caudal border of the mesenteric root, as figured by Frasier ('19) and Vogt ('20). In this way the duodenum comes to lie dorsal to the root of the mesenteric vessels and later acquires with the pancreas a so-called retroperitoneal position.

Now, we may assume that if the coils of the intestine are withdrawn from the umbilical cavity before the duodenal curve is fully formed, its completion may be prevented. This conception has been used to explain various anomalies in the position of the duodenum. Yet, in many of the reported cases such an explanation does not seem to be wholly adequate. This applies to such cases as have been reported by Harman ('98), Bryce ('99), Clermont ('05), Eddy ('12), and Papez ('21), in which a part of the duodenum had passed back of the mesenteric vessels in the usual way and yet an anomalous twist or reversed loop existed in the descending part of the duodenum.

The intestinal anomaly which is the subject of this report is strikingly different from anything that has been presented in the past and provides some special features in addition to those already known which may help to further explain some of the peculiarities of the duodenal and other intestinal anomalies. From the discussion at the conclusion of this paper it will become evident that some of these features add a new factor to the explanations of incomplete rotations of the intestines as usually given.

DESCRIPTION OF CASE

This anomaly occurred in subject 746, male, age sixty-one, from the Niagara County Alms House. Cause of death being given as 'hypertrophy of the heart.' The case resembles the four cases figured by Huntington ('02, figs. 118 to 121), but in his cases the abnormal sac was absent.

The *abdominal viscera* as a whole (plate 1) had a healthy exterior, presenting the smooth glistening appearance usually found in normal subjects. The peritoneal surfaces showed no evidence of any inflammatory process. There was nothing to indicate that the anomaly of the intestines might have been the result of a pathological condition. All of the special features encountered were strongly suggestive of an early embryonic origin of the anomaly.

The *stomach* (*st*), somewhat longer than usual, was of the J-shaped type. The fundus, somewhat constricted from the body, projected to the left and came into relation with the gastric surface of the spleen. The oesophagus (*oes*) above the diaphragm was large and considerably dilated. The position, relations, and peritoneal attachments of the stomach to the liver and spleen were close to normal, excepting that the splenic attachment was at the upper or fundic portion of the mesogastrium. The lower portion of the body of the stomach was ventral to the large upward loop of the colon (*ac*). On the dorsal surface of the stomach the dorsal or caudal layer of the omentum (mesogastrium) was adherent to this loop of the colon. A true gastrocolic ligament, however, was lacking. Back of this adhesion to the colic loop the dorsal or caudal layer of the mesogastrium was attached across the dorsal abdominal wall along the tail and body of the pancreas. Caudal to this there was a short mesocolon supporting the colic loops. The normal fusion between the transverse mesocolon and the dorsal layer of the mesogastrium had not taken place in the usual way.

The *great omentum* consisted of two parts, a right and a left, both attached to the greater curvature of the stomach on the one hand and across the pancreas on the other. The nor-

mal fusion of the caudal layer with the transverse mesocolon was wanting. The right part of the omentum, not represented in figure 1, was attached to the pyloric end of the stomach and extended down along the entire length of the left side of the abnormal sac to which it was adherent. It was supplied by the right gastro-epiploic artery. The left part of the omentum consisted of two short components attached to the body of the stomach, being continuous with the lower border of the gastrosplenic ligament. It was supplied by the left gastro-epiploic artery from the splenic. This part of the omentum covered the upper loop of the unrotated colon, the part which would under normal conditions constitute the right end of the transverse colon. This loop of the colon was situated dorsal to the body of the stomach and was here adherent to the dorsal layer of the left part of the ommental sac. The fusion which normally occurs between the dorsal (caudal) layer of the mesogastrium and the transverse mesocolon had not taken place.

The *duodenum (duo)* was represented by three coils to the right side of the pylorus below the right lobe of the liver. The first part was in the usual position and was attached to the liver by a more or less normal hepatoduodenal ligament containing the usual structures. The descending part formed a sharply bent S-shaped coil whose lower limb occupied a dorsal position in front of the right kidney and there entered the abnormal peritoneal sac to join the jejunum. The duodenum was, therefore, entirely on the right side in an almost completely unrotated condition. That is, the normal curve which the duodenum makes to the left below and dorsal to the embryonic root of the mesentery and its mesenteric vessels had not taken place.

An *abnormal peritoneal sac (sac)* enclosed the coils of the jejunum and ileum. It was lodged within the right side of the general abdominal cavity. The sac separated easily from the parietal peritoneum on its left side, although in places some adhesions had formed. Essentially, however, it was a separate sac whose independence from the parietal perito-

neum, which was partly wrapped around the sac, was evident. Above, the sac was attached to the coils of the duodenum, and behind these, to the peritoneum in front of the right kidney. The left wall of the sac was attached along the intestinal branches of the superior mesenteric vessels. The main mesenteric vessels were not enclosed in the sac. They extended lengthwise along the dorsal midline. The caudal end of the sac was adherent (*ad*) to the caecal end of the colon. This connection (*ad*) was apparently a secondary adhesion, since the wall of the dorsal sac to it extended upward to the mesenteric root.

The right wall of the abnormal peritoneal sac presented on the posterior side an orifice about 2 inches in diameter through which the terminal end of the ileum passed out to join the caecum. The caecum was on the outside of the general peritoneal cavity with the remainder of the colon. Above the orifice the right wall of the sac was drawn in as a partition toward the root of the mesentery. The separation of the sac which it effected was only partial. The upper compartment contained the coils of the jejunum.

Posteriorly, the walls of the sac were reflected over the root of the mesentery some distance in front of the superior mesenteric vessels. Around the proximal part of the mesenteric root remnants of another layer of peritoneum were evident which was in all likelihood the original embryonic layer of the posterior abdominal wall which extended forward over the mesentery. The abnormal sac was, therefore, in the abdominal cavity surrounded in part by parietal peritoneum of the right abdominal wall.

The *jejunum* and *ileum* were enclosed in the abnormal peritoneal sac. They measured $18\frac{1}{2}$ feet in length, the jejunum being about 6 feet long and the ileum about $12\frac{1}{2}$. Both jejunum and ileum were somewhat reduced in caliber, but otherwise their structure was normal. The terminal end of the ileum was narrow (or contracted) for a distance of about 6 inches where it passed out of the orifice of the sac to join the caecum. The thick-walled jejunum occupied the

upper compartment of the sac. The ileum occupied the lower part of the sac. The coils were normal; there were no adhesions; each coil had a freely movable mesentery with normal arterial arcades, lymphatics, and nerves.

The *mesentery* of the small intestine (*mes*) entered the posterior wall of the abnormal sac as a distinct, flat, narrow stalk. This stalk consisted chiefly of the intestinal vascular branches to the jejunum and ileum. The main trunks of the superior mesenteric vessels were behind and outside of the abnormal sac and passed down along the midline adherent to the dorsal abdominal wall in the manner of the normal mesenteric root.

The *large intestine* was of normal length and occupied the left side of the abdominal cavity; that is, it was in the normal greater peritoneal sac. Its position was, therefore, entirely to the left side of the mesenteric root and the abnormal sac. No rotation of the large intestine across the front of the root of the embryonic mesentery had taken place.

The caecum (*cae*) somewhat distended, was situated in the pelvis minor below the lower end of the sac. Here it received the ileum through the orifice in the posterior wall of the sac.

The appendix was about 1 inch in length.

From the caecum the colon (*ac*) extended upward to a position below the pyloric portion of the stomach. It was adherent to the left wall of the abnormal peritoneal sac and was covered in front by the long right part of the greater omentum. In figure 4 this part of the omentum was removed, and the ascending colon was separated from the sac to show the position of the superior mesenteric vessels. This part of the colon was supplied by what would under normal conditions be called the right colic vessels.

The transverse portion of the colon formed a downward loop (*tc*) on the left side extending down to the left iliac fossa and then ascending to form a splenic flexure (*spf*) below the spleen and in front of the left kidney. This loop of the colon was supplied by what, under normal conditions, would be called the middle colic vessels, from the superior mesen-

terics. The splenic flexure and the transverse colon resting against the posterior wall of the stomach were covered by the short left portions of the great omentum. The descending colon occupied the normal position extending from the splenic flexure down to the left iliac fossa and then continuing into a narrow pelvic colon. It was supplied by the inferior mesenteric vessels.

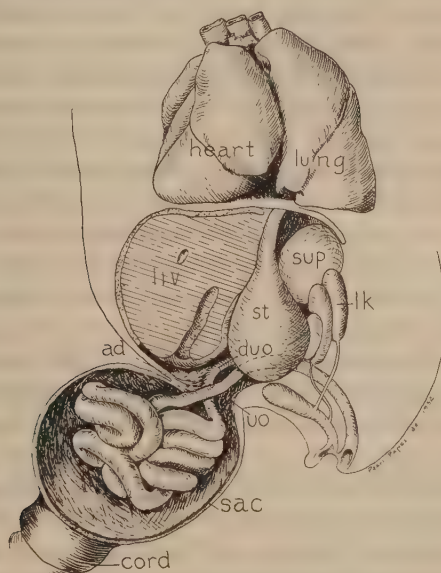


Fig. 1 Reconstruction of a human embryo of 24 mm., showing the position of the small intestines in the umbilical cord constituting a normal umbilical hernia during this stage of development. Modified from F. P. Mall. *Ad* indicates the supposed adhesion which precedes the formation of a right paraduodenal hernia.

The *liver* was somewhat smaller than usual, but presented normal relation and connections.

The umbilical scar was normal.

DISCUSSION

The case here reported represents a peculiar anomaly of the intestines and their peritoneal coverings which can be

explained only by reference to the early embryonic conditions under which the small intestines develop. In the young human embryo up to 30 mm. of crown-rump length the small intestine and the caecum are extruded into the cavity of the umbilical cord, within which they are surrounded by an umbilical sac of peritoneum which forms the lining of this normal umbilical hernia. Within this sac the coils of the small intestine are developed, as has been shown by Mall ('97) and Jackson ('09), producing a bulbous enlargement of the proximal end of the cord. The abdominal orifice of the cord as well as its peritoneal sac do not share in this enlargement, but remain narrow; they surround the distal portions of the duodenum, the embryonic root or pedicle of the mesentery, and the caecal portion of the colon. At this point the peritoneum of the umbilical orifice is brought into intimate relation with the duodenum and mesenteric root, and it is probable that in the present case an actual adhesion and fusion took place between the upper portion of the peritoneal orifice and the duodenum and mesentery. The possibility of this is further supported by the presence of a peritoneal fold which has been shown by Lewis ('12, Keibel and Mall, p. 320, fig. 242) to extend from the cephalic border of the umbilical orifice inward toward the duodenum. We may assume that in the present case a firm adhesion took place between this fold of the peritoneal orifice and the duodenum before the intestines were retracted from the umbilical cord which normally takes place when the embryo has reached about the 30-mm. crown-rump length. When the withdrawal occurred in the present specimen we may assume that the peritoneum of the umbilical cord was separated from the interior of the umbilical cord by the tractions exerted at the point of duodenal adhesions and was drawn into the abdomen with the small intestines. Since the linea alba and the umbilical scar were normal, it does not seem probable that any layer other than the peritoneum of the umbilical cord was pulled in. Within the abdominal cavity the small intestines grew, expanding the abnormal sac to its adult dimensions. That

this is the probable explanation is further supported by the fact that the sac gave the impression of being extraperitoneal, so far as the general peritoneal lining was concerned. It received on its sides a partial covering of the parietal peritoneum of the anterior abdominal wall. The peritoneum of the caudal part of the umbilical orifice which enclosed the caecum remained open (unadherent) so that the large intestine withdrew completely from the umbilical peritoneal sac.

Owing to the adhesion of the umbilical orifice to the duodenum, the duodenum was prevented from rotating around the embryonic root and the usual duodenal curve was not formed. The rotation of the colon to the right of the embryonic root across the ventral side of the duodenum was prevented by the same condition. Hence, the entire colon and its mesocolic attachments remained on the left side.

From this case it can be seen that the size of the umbilical orifice and its intruding peritoneal fold may play an important part in some cases in the retardation or arrest of the normal rotation of the intestines around the embryonic root of the mesentery even when the normal factors which cause the withdrawal of the umbilical viscera are sufficient to do so.

RELATION TO THE PARADUODENAL HERNIAS

Since the foregoing description was written, my attention has been called by Dr. E. A. Boyden to the possible relation of the concealed umbilical hernia here described to the so-called retroperitoneal or paraduodenal hernias of clinical literature.

Retroperitoneal hernia was first described by Treitz ('57); since then a voluminous literature has grown up on the subject. Moynihan ('96 and '06) was the first English author to organize the knowledge on the subject in his book on 'Retroperitoneal hernia.' He collected seventy-four cases from the literature. Since then a complete bibliography and analytic tables of cases reported (from 1906 to 1923) have been given by Andrews ('23). Dowdle ('32), in a recent paper, has added cases reported since 1923. All told, 34 cases of

right and 105 cases of left paraduodenal hernias have been recorded, and a number of other doubtful cases not included are probably instances of this same anomaly.

The many excellent illustrations and descriptions which occur in literature make clear the appearance of the paraduodenal hernias as they are seen on the operating table or in the course of a dissection or postmortem examination. The small intestines in the majority of cases are the only structures enclosed in a special peritoneal sac which is suspended either from the transverse colon or the region of the duodenum, the former being the most common condition (left paraduodenal hernia). They are classified as right and left paraduodenal hernias. However, the classification depends in most cases on the position of the orifice of the hernial sac, its relation to the hernial mass, to the root of the mesentery, and to the adjacent folds and blood vessels.

Moynihan ('06, 2nd ed.) believed, along with others, that these hernias arose as a result of the intrusion of a loop of the small intestine into one of the nine peritoneal fossae which occur near the duodenojejunal flexure or in relation to the mesenteries. These fossae are named as follows: 1) the superior duodenal fossa; 2) the inferior duodenal fossa, which with the foregoing one makes up the fossa of Treitz; 3) the paraduodenal fossa of Landzert; 4) the mesocolic fossa; 5) the mesentericoparietal fossa of Waldeyer; 6) the posterior duodenal fossa of Gruber; 7) the recessus intermesocolicus transversus; 8) the duodenojejunal fossa; and, 9) the infra-duodenal fossa.

The theory advanced by Moynihan was that these fossae are formed by fusion folds originating in foetal life by the process of physiological adhesions first described by Toldt ('89). However, these fossae are not independent or constant entities, but variants resulting from the manner in which the peritoneal adhesions take place in this region during development. One or more of these might, therefore, be present in any one individual, but not all. Moynihan believed that insinuation of the gut into fossae 1 and 2 and their combina-

tions produced a left paraduodenal hernia; into fossa 4 produced a right paraduodenal hernia; into fossa 5 produced a mesocolic hernia. The remaining fossae are not considered of practical importance.

The interpretations of Moynihan have been followed by most authors. In the majority of cases the classification of the hernia and the position of the orifice of the abnormal peritoneal sac and its relation to blood vessels have received most attention, the assumption being that the hernia arose by an intrusion of the gut into one of the peritoneal fossae.

Misgivings have arisen concerning this mode of origin. Since no one has ever observed a paraduodenal hernia in the process of formation either in the adult or in the fetus, and since the hernia is always total or subtotal and never contains any other viscus than the small intestine, its mode of formation is still considered uncertain.

Nagel ('23), of the Mayo Clinic, says:

The origin of right paraduodenal hernia has not been definitely established since it has not been observed in the early and progressive stages. . . . Nearly all observers agree that the condition represents a true herniation of the intestines into the one or the other of the fossae about the duodenal flexure.

Lower and Higgins ('25) state that the embryonic formation of 'fusion folds' is not accepted by other observers as the origin of the fossae into which herniation occurs; but they themselves adhere to this view. They do not believe that the vessels are primary factors in the formation of the folds. In fact, this vascular relation has never been satisfactorily established.

Andrews ('23) in particular voices a number of objections, excerpts from which follow:

Since the publication of Moynihan's book (1906) as each case has been discovered and published, the same mechanism has been assumed and the same authors and theories quoted in a multitude of papers. The absurdity and grotesqueness of this whole conception must be evident from a consideration of the following facts: 1) Differential pressure is utterly lacking . . . the pressure in the main cavity can never rise higher than within the pouch. Any vis a tergo to

account for the formation or growth of such a hernia is totally absent. 2) There are literally hundreds of similar folds and fossae in the peritoneum many of which are of much greater size, and they are practically never the sites of such 'hernias.' 3) In all but a very small number of cases reported the degree of herniation has been total or subtotal. How can one conceive of a force which would, once begun, practically always continue to act until the gut has been segregated into a sac, even when the rest of the belly was empty?

If the hernia is of embryonic or fetal origin, it is hard to see how the developing gut, practically functionless so far as peristalsis is concerned, could insinuate itself into one of the small adhesion folds. In fact, an entirely different mechanism would have to be postulated.

Andrews concludes that the so-called paraduodenal hernia is a congenital anomaly due to the imprisonment of the small intestines beneath the mesentery of the developing colon, but even this is not adequate to account for all the conditions usually seen.

It seems to me that the right and left paraduodenal hernias and their surrounding sac have a more natural origin in the very early embryonic umbilical hernia (fig. 1). When the small intestines are drawn into the abdominal cavity, they also draw in with them their surrounding peritoneal umbilical sac, due to a premature adhesion of its orifice to the duodenal coils and to the mesenteric root. In case the large intestine has been fully rotated to the right side across the duodenum and mesenteric root before the adhesion of the orifice of the sac and retraction of the gut had taken place, it would produce the more common form or left paraduodenal hernia (fig. 2) in which the abnormal sac is situated to the left of the ascending colon and is suspended from the transverse colon and its mesentery. The cases illustrated by Desjardins ('18), Novak and Sussman ('24), and others are good examples. In case the large intestine has not completed its rotation before the adhesion of the orifice of the sac and retraction of the gut had taken place, the condition produced would be a right paraduodenal hernia (fig. 3), in which the abnormal sac is situated to the right of the ascending colon. The case re-

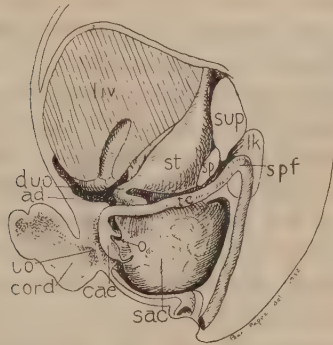


Fig. 2 A diagrammatic sketch representing the formation of a left paraduodenal hernia in which the colon has crossed over to the right of the hernial sac, in an embryo of about 30 mm. This is the more common type of paraduodenal hernia. Note that the peritoneal sac which surrounds the small intestines has been withdrawn from the umbilical cord leaving the umbilical orifice (*uo*) and ventral abdominal wall stripped of its original peritoneal covering. The duodenum and terminal ileum are surrounded by a peritoneal orifice (*o*) which originally occupied the orifice of the umbilical cord.

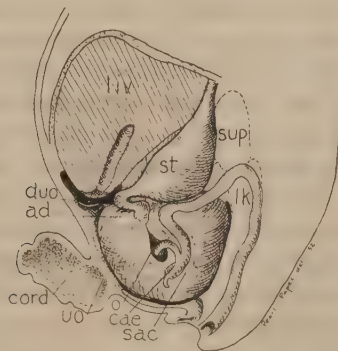


Fig. 3 A diagrammatic representation of the formation of a right paraduodenal hernia in which the entire colon remains on the left side of the hernial sac, in an embryo of about 30 mm. This is the less frequent type of paraduodenal hernia. Note that the peritoneal sac surrounding the small intestines has been withdrawn from the umbilical cord leaving the umbilical orifice (*uo*) and ventral abdominal wall stripped of its peritoneal covering. The duodenum and terminal ileum are surrounded by a peritoneal orifice (*o*), which originally occupied the orifice of the umbilical cord.

ported in this paper would be a right paraduodenal hernia. The cases figured by Nagel ('23), Lower and Higgins ('25) and others would be of the same nature.

This classification would differ from the older one by assuming an identical origin for both right and left hernias from the early umbilical peritoneal sac. In case the colon has completed its rotation over to the right of the sac, it would result in a left hernia suspended in part from the transverse mesocolon (fig. 2). In case the rotation of the colon was incomplete, it would result in a right hernia suspended from the duodenum, to the right of the ascending colon which remains on the left side of the sac (fig. 3).

In addition to the objections already stated to the theory of formation by way of the paraduodenal fossae, the mechanism here postulated is supported by the following considerations: 1) There is always a normal umbilical hernia in the early embryo, which hernia is normally reduced by the withdrawal of the gut into the abdominal cavity through the umbilical orifice (at the 30-mm. stage). 2) A peritoneal sac surrounds the normal umbilical hernia and presents a narrow abdominal orifice (fig. 2, *uo*). 3) It is here assumed that an adhesion (*ad*) of the cephalic (upper) border of this orifice to the duodenum and adjacent structures may take place before withdrawal of the gut, in which event the umbilical peritoneal lining is drawn in with the gut. 4) In the adult, in either right or left hernia, the adhesion or suspension of hernial sac (*ad*) is at the upper end around the mesenteric root. 5) The mesentery of the small intestines extends into the sac through its posterior wall, greatly modifying its posterior abdominal wall fixations in its distal parts. 6) The orifice of the abnormal sac (*o*) is in its posterior wall below the point of suspension, either on the right side or the left side or near the lower end, depending on the position of the caecum. 7) The jejunum and ileum are totally enclosed, only a small part of the ileum passes out of the orifice to join the caecum. 8) The orifice (*o*) is not related to any fold formed by vessels of the posterior abdominal wall, but terminals of other vessels may occur around it.

SUMMARY

The foregoing article describes and figures a concealed umbilical hernia in a dissecting-room cadaver. The small intestines were enclosed in a special abnormal peritoneal sac situated within the general peritoneal cavity. The sac was attached above to the duodenum; posteriorly it was invaded by the mesentery of the small intestines. An orifice about two inches in diameter at the lower end of the sac, situated in its posterior wall allowed the free exit of the terminal ileum. The colon was situated to the left side of the abnormal sac excepting the caecal end, which was below the sac in relation to the orifice.

The anomaly is interpreted as one of early embryonic origin by assuming that the abnormal peritoneal sac was derived from the peritoneum of the normal umbilical hernia which encloses the small intestines prior to the 30-millimeter stage of embryonic growth. It is believed that the neck of the sac at the umbilical orifice became adherent to the duodenum by virtue of the close apposition which exists between them at this early period of development. The retracting forces which suddenly draw the intestines into the abdominal cavity also draw in the peritoneum of the early embryonic cavity in case an adhesion happens at the umbilical orifice. This mechanism in the formation of such intra-abdominal hernias is illustrated by three figures in the text.

A review of the clinical literature convinces the author that the right and left paraduodenal (or retroperitoneal) hernias in the vast majority of instances probably arise in this manner and not as has been postulated by Moynihan and many others by insinuation of the gut into or inclusion under one of the paraduodenal fossae or folds. A number of objections to the latter theory of origin are cited and various arguments in favor of an origin from the early umbilical hernia are stated. The formation of the right paraduodenal hernias is explained by assuming that the adhesion took place before the colon had completed its normal rotation over the duodenum and root of the mesentery thus leaving the abnormal

sac to the right of the colon. The formation of the more common left paraduodenal hernias is explained by assuming that the colon had completed its rotation over the adhesion or before the adhesion had occurred, thus bringing the abnormal sac between the ascending and descending portions of the colon suspended above from the transverse colon.

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PLATE 1

EXPLANATION OF FIGURE

4 Intestinal anomaly in subject 746, Cornell collection. In the figure the two parts of the great omentum described in the text have been cut away so as to expose more fully the configuration of the intestines and the abnormal peritoneal sac.

ABBREVIATIONS

<i>ac</i> , ascending colon	<i>o</i> , orifice of hernial sac
<i>ad</i> , adhesion	<i>oes</i> , oesophagus
<i>cae</i> , caecum	<i>om</i> , cut edge of omentum
<i>duo</i> , duodenum	<i>pc</i> , pelvic colon
<i>fl</i> , falciform ligament	<i>py</i> , pylorus
<i>gb</i> , gall bladder	<i>rl</i> , round ligament
<i>hd</i> , hepatoduodenal ligament	<i>sac</i> , abnormal peritoneal sac
<i>ic</i> , ileocolic vessels	<i>sp</i> , spleen
<i>il</i> , ileum	<i>spf</i> , splenic flexure
<i>j</i> , jejunum	<i>st</i> , stomach
<i>liv</i> , liver	<i>sup</i> , suprarenal gland
<i>lk</i> , left kidney	<i>tc</i> , transverse colon
<i>mes</i> , mesentery	<i>uo</i> , orifice of umbilical cord
<i>mv</i> , mesenteric vessels	



POTENCIES OF THE END BUD AND OTHER CAUDAL LEVELS OF THE EARLY CHICK EMBRYO, WITH SPECIAL REFERENCE TO THE ORIGIN OF THE METANEPHROS

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THREE FIGURES

INTRODUCTION

In 1926, Willier reported results of transplanting the entire pellucid area of young chick blastoderms to the chorio-allantoic membrane, to determine if gonads would differentiate in the absence of the primordial germ cells. In this, and in a subsequent detailed report (Willier and Rawles, '31) of transplants of whole blastoderms of presomite and early-somite stages, it was shown that all anterior structures back to and including the level of the mesonephros were differentiated in these grafts. In his studies on the rôle of Hensen's node in the formation of the chick embryo, Hunt ('31) has demonstrated that the isolated node level of the primitive-streak stage has almost as great capacity for differentiation as that shown by whole blastoderms of primitive streak, head-process and early-somite stages. There was no indication of differentiation of metanephros in either type of isolation, even in those from donors with twelve somites, the oldest stages used. The node level appeared to be totipotent for all of the embryo anterior to a certain level of the trunk, just in front of the metanephros. The failure of metanephros, the only criterion of differentiation of caudal levels of the embryo, to appear in these grafts led to the experiments outlined in this paper.

It was noted, especially in transplants of the node level, that the capacity to produce mesonephros increased as the node moved posteriorly. This was indicated by the greater frequency with which mesonephros was found in grafts from early-somite stages than in grafts from presomite stages. The presence of two additional structures, gonad and adrenal gland, in grafts of somite stages was also indicative that the node level acquires potentiality to form more posterior structures as it moves backward. On the basis of these results, it was predicted (Willier, '31) that, after the node had moved posteriorly to a certain level of the primitive streak, it would acquire the potency of metanephros also. This prediction was made with the reservation that certain other relations might be established, subsequent to those primarily set up by the node, which would be necessary for metanephros to differentiate.

About the eighteen- to twenty-somite stage, the node, which has become quite small, begins to increase in size very rapidly and forms, by the twenty-one- or twenty-two-somite stage, a prominent knob-like swelling, the end bud. On the basis of the preceding discussion, one might expect that the realization of capacity to form metanephros might accompany this change in the node.

Holmdahl ('26) has attributed considerable significance to the end bud. He believed that all of the embryo posterior to approximately the twenty-seventh or twenty-eighth somites is derived from material of the end bud, which he regarded as being composed of indifferent material. If this conception is true, we would expect metanephros to be formed from material proliferated from the end bud, since it lies in the region supposedly formed from this structure.

It was with this point in mind that experiments were carried out to determine the capacity of the end bud for differentiation of caudal structures. An analysis was first made, by means of chorio-allantoic grafts, of the differentiating capacity of the end bud and short primitive streak behind it; and, contrary to expectations, metanephros and, still more

surprising, neural tube and notochord did not differentiate in these grafts.

This led to a series of experiments to determine, if possible, what conditions must be set up in the posterior part of the embryo before metanephros will differentiate in grafts. Since the metanephros arises from two primordia, there exists the possibility of a dependent differentiation. Such a dependence had been postulated by Boyden ('28), whose experiments indicate that differentiation of the nephrogenous tissue is dependent on ureter formation. An analysis of this problem by transplantation of the metanephric primordia to the chorio-allantoic membrane supports such a conception.

It affords me much pleasure to express my sincere appreciation to Dr. B. H. Willier, to whom I am indebted for the suggestion of the problem and for helpful advice and criticism.

MATERIAL AND METHODS

The method used in these experiments was essentially that described by Willier ('24). After the donor eggs had been incubated to the desired age, the blastoderms were removed from the yolk and placed in a Petri dish containing warm saline solution. Before the desired levels were removed from the blastoderm, the exact stage of the donor, as indicated by the number of pairs of somites present, was determined. Great care was taken in making this somite count, since it was essential to know the exact stages of the donors used in all of the experiments. It was not sufficient to know the hourly age of the donors, for, as indicated in the tables presented in this paper, there is no very close relationship between age and somite number.

As soon as the stage of the donor had been determined, the level of the embryo desired for transplantation was isolated by carefully planned cuts. In the cases where the entire pellucid area of a certain level of the embryo was transplanted, the transections were made with a small iris-knife.

Finely ground steel needles were used to separate the minute metanephric primordia from the embryo.

The isolated part was then sucked up into a fine glass pipette, which had been drawn out at the tip to a diameter suited to the size of the implant. By means of the pipette, the graft was placed in contact with a blood vessel of the chorio-allantoic membrane of an eight- or nine-day host embryo. The host had been previously prepared by having a small rectangular window sawn in the shell over the blood vessel, which had been located by candling.

After a period of nine or ten days on the host membrane, the grafts were removed, fixed in Bouin's fluid, sectioned at 8μ , and stained with iron haematoxylin.

In connection with these experiments, a number of preparations of normal embryos were examined. These included whole mounts and serial sections of end-bud stages, with from eighteen to twenty-six somites, and serial sections of representative stages from this latter stage up to fifty-one somites. Besides my own preparations, I had the opportunity to examine a very fine set of serial sections of every somite stage between the eighteen- and fifty-one-somite stage, so kindly lent to me by Miss Mary Rawles, of this department.

White Leghorn eggs were used almost exclusively in these experiments.

DIFFERENTIATION CAPACITY OF GRAFTS OF END-BUD AND
PRIMITIVE-STREAK LEVELS OF THE EMBRYONIC
AXIS OF END-BUD STAGES

Transplants were first made of the end-bud and primitive-streak levels, with the idea that the former might possibly have the capacity to form metanephros. As has been previously pointed out, Hensen's node level of blastoderms up to the twelve-somite stage is not capable of differentiating into metanephros. It was believed, however, that it was not unlikely that the node level would acquire the capacity to form kidney after it had enlarged to form the end bud.

Before we proceed with an account of the experiments, it will be well to give a brief description of the events associated with the formation of the end bud in normal development. As the node moves posteriorly along the primitive streak, leaving in its wake notochord and the floor of the neural tube, as Wetzel ('29) has so clearly shown by his marking experiments, it becomes smaller and smaller, reaching its minimum size by the seventeen- or eighteen-somite stage. At this time the neuropore is still open, so that the node is partially enclosed by the presumptive neural-tube material, the rhomboidal sinus. Very soon after this stage, the node becomes a center of very active cell proliferation, and as a result forms a prominent knob-like swelling at the posterior end of the embryonic axis which is known as the end bud ('Nodus caudalis,' Rauber, '80; 'Schwanzknöpfchen,' Braun, '82; 'Rumpfknospe,' Keibel, '10; 'Endwulst,' Holmdahl, '24; 'Endknopf,' Wetzel, '29). End-bud formation is in progress by the eighteen- or nineteen-somite stage, and the end bud is fully formed by the twenty-two- or twenty-three-somite stage (fig. 1). Accompanying the enlargement of the node is the closure of the neuropore, so that the end bud is situated behind the closed neural tube, and the rhomboidal sinus is no longer apparent.

Behind the end bud is the short primitive streak which exists, as such, until the tail fold appears about the twenty-five-somite stage. At this time the end bud comes to lie at the most posterior end of the embryonic axis, the primitive streak turning in under as the folding occurs, and becoming so closely allied to the end bud as to lose its identity as a streak. From this time on, the end bud and the remnant of the primitive streak are situated at the tip of the developing tail bud.

It was not unreasonable to believe that an accompaniment of this sudden increase in activity on the part of the node might possibly be the acquisition of further potencies, especially those of the metanephros. This belief was strengthened by the fact that Holmdahl, Schumacher, and others have

attributed great significance to the end bud in the formation of the posterior part of the embryo.

To determine to what extent potencies are fixed in the end bud and primitive streak, levels of the blastoderm containing these structures were transplanted to the chorio-allantoic membrane. Transplants of three types were made: 1) the end-bud and primitive-streak levels; 2) the end-bud level alone; 3) the primitive-streak level alone.

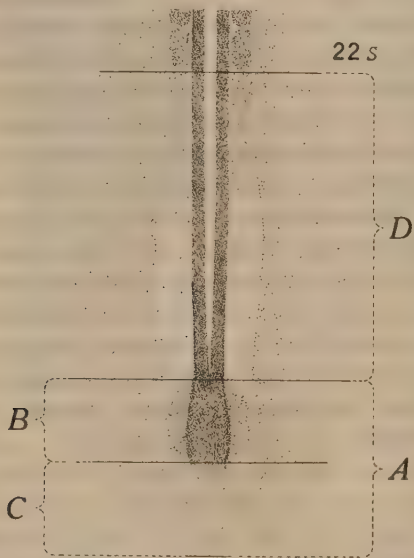


Fig. 1 Camera-lucida drawing of the posterior end of a twenty-two somite embryo. The levels isolated, A, B, C, and D, are described in the text. The solid lines indicate where the transections were made.

I. Grafts of the level including both end bud and primitive streak

These grafts included all of the pellucid area of the end-bud and primitive-streak levels, indicated as level A, figure 1. Transplants were taken from donors varying from the seven-teen-somite stage, at which time the node is at its minimum size before its enlargement to form the end bud, up to the

thirty-somite stage, when the end bud and primitive streak are at the tip of the developing tail bud.

The structures differentiated in these grafts are indicated in table 1. It is clearly shown that there was no differentiation of any structure characteristic of a specific level of the embryo. The only structures appearing were of a highly generalized type, found in grafts of any level and not indicative of any specific potentiality. Metanephros was not present in any case, and, still more unexpected, neural tube and notochord were not differentiated in a single graft.

With the exception of five of the twenty-three grafts in which there appeared a few scattered ganglion cells, there was only one graft (127-8), from an eighteen-somite donor, in which there was indication of nervous tissue. In this graft there was a small area of nervous tissue which was not organized into a neural tube with a lumen and epithelium, and was not accompanied by notochord. It is probable that in this case a small amount of rhomboidal sinus was included with the material isolated, for at this stage the node is to some extent bounded laterally by presumptive medullary plate. An effort was made, at the time of isolation, to include as little of this material as possible. In stages later than this, the end bud lies posterior to the closed neural tube, and this difficulty was not encountered. The presence of ganglion cells in five of the grafts appears to have little significance, since these cells appear in grafts of many levels in which there is no evidence of central nervous system.

As noted in the table, gut, smooth muscle, and integument appeared most frequently, while cartilage, skeletal muscle, and feather germs were present in less than one-third of the cases. The grafts were all quite small and in many cases the bulk of the graft was mesenchyme, the differentiated tissues comprising only a small part. Differentiation was usually comparatively poor, i.e., the gut which appeared in these grafts, for example, was not characteristic of gut which appears in posterior levels. The largest grafts obtained from this level were no more than 4 mm. in length. This is approxi-

mately one-sixth to one-eighth of the size of the grafts obtained by Hunt ('31) from isolations of Hensen's node level of primitive-streak stages.

Successful grafts were obtained from twenty-three of the forty-four hosts which remained alive up to the time of removal of grafts. In the other twenty-one cases, the grafts failed to become incorporated or were so poorly incorporated as to contain only mesenchyme. It is of interest to note that the percentage of successful grafts (52 per cent) is considerably lower than that obtained by Hunt from transplants of the node of primitive-streak stages (73 per cent) and of head-process stages (60 per cent).

II. Grafts of the end-bud level alone

The level B, figure 1, was isolated to see if the end-bud level differed in capacity for differentiation from the primitive-streak level behind it. Since the end bud and primitive streak together had shown so little capacity, we hardly expected different results in this case. The results of these transplantations are so uniform, and agree so well with the previous experiment, that it is unnecessary to arrange them in tabular form.

With the exception of one case, axiate structures were not present. In this one graft (DD-4), from a seventeen-somite donor, some rhomboidal sinus material was intentionally included with the node, and as a result well-defined neural tube and notochord were present. In the other grafts special care was taken not to include any of the presumptive medullary material.

Eleven grafts of this level, from donors varying from seventeen- to twenty-five-somite stages, were sectioned and examined. These eleven grafts were obtained from twenty-nine hosts which were alive at the end of the experimental period, showing only 38 per cent success. The grafts were very small and the differentiation was limited, for the most part, to small areas of gut, smooth muscle, and integument. Nine of the eleven had gut and muscle, the other two consist-

ing of integument only. The single case in which cartilage appeared was in the afore-mentioned DD-4. Integument appeared seven times and feather germs twice in the eleven grafts. It is apparent, therefore, that this level has the capacity to produce only gut, smooth muscle, and integumentary structures.

III. Grafts of the primitive-streak level alone

Isolations were made which included all of the pellucid area posterior to the end bud, indicated as level C, figure 1. There were eleven successful grafts, taken from seventeen-somite up to twenty-five-somite donors, resulting from the twenty one cases in which the hosts were alive (52 per cent successful). The results are very little different from those of the end bud level. Four of the grafts have nothing but integument, one of the four having a few feather germs. Five showed differentiation of gut, only three of which have muscle associated with it, while two of the five had skin present. One graft showed only muscle, while the eleventh had muscle and skin. Cartilage was not present in any case.

EXPERIMENTS CONCERNING THE DIFFERENTIATION OF THE METANEPHROS

It has been shown that metanephros does not differentiate in grafts of Hensen's node or primitive-streak levels of the early chick blastoderm (Hunt, '31); in grafts of whole blastoderms up to twelve-somite stage (Willier and Rawles, '31); or in grafts of the end-bud and primitive-streak levels up to the thirty-somite stage. At what stage, then, is it possible to obtain differentiation of metanephros in grafts, and what are the factors that determine its differentiation? As suitable conditions apparently do not exist in the node or end-bud levels, it seemed likely that differentiation would result from transplantation of either, 1) the region between the last differentiated somite and the end bud, or 2) the level of the embryo in which the primordia of the metanephros arise.

It is necessary to give a short account of the normal development of the metanephric primordia before we proceed with the analysis. The metanephros forms from two primordia, the secreting tubules and renal corpuscles arising from the nephrogenous tissue of the thirty-first, thirty-second, and thirty-third somites, while the ureter and collecting tubules are formed from the metanephric diverticulum—an evagination from the wolffian duct at its posterior end. The wolffian duct, arising in the anterior part of the body, grows posteriorly, usually keeping pace with the formation of somites, until it reaches the level of the cloaca. It reaches its most posterior extent about the thirty-five somite stage, and enters the cloaca at the level of the thirty-fourth or thirty-fifth somite, about the forty-somite stage. The metanephric diverticulum is formed from the wolffian duct near its point of union with the cloaca, and grows anteriorly where it becomes closely associated with the metanephrogenous tissue. Differentiation of this metanephric blastema into secreting tubules and renal bodies takes place following its contact with the metanephric diverticulum which is differentiating into the collecting elements of the kidney.

It is evident that to include both primordia of the kidney in an isolated level of the embryo, it is necessary to have the region between the thirty-first and thirty-fifth somites, inclusive. Following the failure of metanephros to develop in grafts of the region between the last differentiated somite and the end bud, which were made as the first step in the analysis of the factors necessary for the differentiation of the metanephros, grafts were made of the thirty-one- to thirty-five-somite level, inclusive. A third series of experiments was then made, in which the metanephrogenous tissue, together with the section of the wolffian duct from which the metanephric diverticulum arises, were isolated and transplanted. The results of these three series of experiments are given below.

I. Grafts of the level between the last differentiated somite and the end bud

These grafts were made in view of the possibility that the presumptive metanephric material might be located in the region in front of the end bud, and be laid down by elongation of this region instead of being formed from the material of the end bud. If the segmental plate should elongate to any extent during somite formation, more somites would be formed from it than one would estimate by measuring it at a given stage.

Grafts were made of all of the pellucid area between the last differentiated somite and the end bud (level D, fig. 1) from donors ranging from eighteen somites to twenty-six somites. If no elongation of this region occurs during somite formation, it is estimated that it would form approximately six somites. Even if only six somites form from this region, it is seen that some of the presumptive metanephric level will be formed from this region of the older donors used.

Table 1 indicates the structures differentiated in the fifteen successful grafts obtained from twenty-five hosts. Only those structures that one would expect from a graft of this level were found. Metanephros did not occur in any case. Mesonephros was present in eleven of the fifteen grafts, and there was no question as to its identity, thus ruling out any doubt as to the presence of metanephros.

It is pertinent at this point to discuss the differences existing between meso- and metanephros by which we were able to differentiate between the two in grafts. While there are a number of minor differences, which taken as a whole are convincing enough, our final criterion of metanephros was the presence of a ureter which was found to be in union with the collecting tubules. In the case of mesonephros, the wolffian duct was always in connection with the collecting tubules.

It was found upon examination of grafts of the entire wolffian ridge of five-day donors, in which both metanephros and mesonephros occurred, that we could distinguish a very characteristic difference in the histology of the wolffian duct

and ureter. The wolffian duct has a very distinct, high columnar epithelium at all levels. Near the point where the wolffian duct connects with the collecting tubules, the epithelium is simple columnar, the cells being very high. At other levels, the wolffian duct is characterized by a columnar epithelium in which the nuclei are arranged in several layers. The epithelium of the ureter, on the other hand, is very rarely columnar at any level. Near its point of division to form collecting tubules, the ureter has simple, cuboidal epithelium. As we follow the ureter toward the cloaca, we seen that its epithelium becomes stratified, but is never columnar, being, instead, a so-called transitional type. The epithelium of the ureter is thrown into folds so that the stratification varies in thickness, in any cross section, from one cell to five cells. The epithelium of the wolffian duct, on the contrary, is of a uniform thickness at any particular level. The epithelium of the ureter is surrounded by a wide band of flat cells which form the tunica propria. This band is, in most cases, considerably wider than that found about the wolffian duct.

By means of these features, we were able to distinguish readily between ureter and wolffian duct in our grafts, and so tell with certainty which type of nephric tissue was present. Besides this criterion, there were others by which we could distinguish one type from another. The tubules of the mesonephros are usually less closely bound together by interstitial tissue, giving the mesonephros the appearance of being much less compact than the metanephros. This furnishes a difference which is usually readily apparent. The tubules and glomeruli of the metanephros are uniformly smaller in size than those of the mesonephros. Metanephros, at this stage, usually contains a considerable amount of undifferentiated nephrogenous tissue, a feature never apparent in the case of mesonephros. On the basis of these differences, we feel justified in stating whether meso- or metanephros was present in any particular graft.

Besides mesonephros, we found neural tube, notochord, gut, muscle, cartilage, skin, and feather germs in a large majority

of the grafts of this series. Gonad and adrenal glands were found in only two grafts, which is a rather low percentage, considering that these bodies normally arise at this level.

II. Grafts of the level of the blastoderm in which the metanephric primordia normally arise

Since metanephros did not form in any of the previous types of grafts, we now turned to the level of the embryo in which the primordia arise. We may divide the series of grafts, listed in table 2, into two groups, 1) those from donors younger than the thirty-five-somite stage; these grafts from isolations including the presumptive metanephrogenous tissue and a section of the wolffian duct, but not the specific level of the duct which gives rise to the ureter; 2) the thirty-one- to thirty-five-somite level, which includes both the metanephrogenous tissue and the specific ureter-forming level of the wolffian duct.

The results of these experiments are so uniform as to render it unnecessary to consider these two groups separately from this point on. A total of thirty-three grafts were sectioned and examined. These were taken from donors between the twenty-seven- and fifty-one-somite stages. The grafts were quite large in most instances, since part of the hind-limb level was included, this resulting in large sections of limb cartilage and bone. Since we are concerned only with the metanephros, it will suffice to say that the grafts up to the fifty-somite-stage showed, uniformly, differentiation of only neural tube, notochord, cloaca, cartilage, bone, integument, feather germs, smooth and skeletal muscle. We first identified cloaca by the fact that wolffian ducts and ureters joined with it. We had no other way of distinguishing it from hind gut in the grafts. The epithelium was of a stratified columnar type, which was non-specific as far as we could tell. Mesonephros was found in eight of the grafts. In every case, the mesonephros consisted only of a few tubules, which were, however, so evidently mesonephric as to leave no doubt as to their identity, using the criteria already described. This differenti-

TABLE 2

Structures differentiated in grafts of the level of the embryo in which the metanephric primordia arise; and in grafts of the metanephric primordia

SERIAL NUMBER OF GRAFT	LEVEL OF THE BLASTODERM GRAFTED	STAGE OF DONOR (SOMITE NUMBER)	AGE OF DONOR (HOURS)	METANEPHROS														
				MESONEPHROS	WOLFFIAN DUCT					OLOACA	SPINAL CORD	NOTOCHORD	CARTILAGE	BONE	INTEGUMENT	FEATHER GERMS	MUSCLE	
						Secreting tubules	Glomeruli	Collecting tubules	Ureter									
F-1	Posterior to 27s.	27	60							+				+				
J-6	Posterior to 29s.	29	61							+	+	+	+					
C-2	Posterior to 29s.	29	59							+			+					
E-8	Posterior to 30s.	30	64								+	+	+					
G-11	Posterior to 29s.	31	64							+	+	+	+					
B-2	Posterior to 31s.	31	61							+			+					
J-3	31s. to 32s. (inclusive)	32	60							+			+					
C-7	Posterior to 30s.	33	60							+	+	+	+					
K-2	31s. to 33s. (inclusive)	33	60							+			+	+				
J-10	30s. to 34s. (inclusive)	34	61							+	+	+	+					
F-9	30s. to 33s. (inclusive)	34	63	+	+					+			+					
D-1	Posterior to 30s.	35	60							+	+	+	+					
J-5	31s. to 35s. (inclusive)	35	61							+	+	+	+					
H-6	30s. to 33s. (inclusive)	37	63	+	+					+	+	+	+					
AA-2	30s. to 35s. (inclusive)	40	69	+	+					+	+	+	+					
Z-9	30s. to 35s. (inclusive)	40	70	+	+					+	+	+	+					
X-7	30s. to 38s. (inclusive)	42	71	+	+					+	+	+	+					
X-8	31s. to 38s. (inclusive)	42	71							+	+	+	+					
Z-7	30s. to 35s. (inclusive)	43	70	+	+					+	+	+	+					
O-5	30s. to 35s. (inclusive)	44	72							+	+	+	+					
117-4	31s. to 34s. (inclusive)	45	84	+	+					+	+	+	+					
122-1	29s. to 35s. (inclusive)	45	93							+	+	+	+					
122-3	31s. to 35s. (inclusive)	46	93							+	+	+	+					
117-5	31s. to 35s. (inclusive)	46	84	+	+					+	+	+	+					
125-1	31s. to 35s. (inclusive)	47	94							+	+	+	+					
125-2	31s. to 35s. (inclusive)	47	94							+	+	+	+					
122-4	31s. to 35s. (inclusive)	48	94							+	+	+	+					
122-5	31s. to 35s. (inclusive)	48	94							+	+	+	+					
122-6	31s. to 35s. (inclusive)	48	94							+	+	+	+					
126-3	31s. to 35s. (inclusive)	49	95							+	+	+	+					
BB-4	30s. to 34s. (inclusive)	50	94					+	+	+	+	+	+					
BB-3	30s. to 34s. (inclusive)	51	95					+	+	+	+	+	+					
BB-6	30s. to 35s. (inclusive)	51	95					+	+	+	+	+	+					
QQ-6	M (left)	36	74	+	+					+								+
LL-3	M (left)	40	72							+	+							+
MM-2	M (left)	40	73	+	+													
QQ-8	M (right)	42	75							+	+							+
111-3	M (right)	45	90							+	+							
104-1	M (right)	46	92		+					+	+							
103-2	M (right)	46	92							+	+							+
103-1	M (left)	47	93		+					+	+							+
101-1	M (left)	48	93		+	+	+	+	+	+	+							+
109-7	M (right)	48	95							+	+							+
106-2	M (right)	49	94		+	+	+	+	+	+	+							+
111-2	M (left)	50	94							+	+							+
107-4	M (left)	50	98		+		+	+	+	+	+							
106-4	M (left)	51	98				+	+	+	+	+							
107-2	M (right)	51	99							+	+							+
107-1	M (right)	51	98							+	+							

ation of mesonephros may have been due to the fact that there probably is not a sharp boundary between meso- and metanephros, at least the nephrogenous tissue of the transition zone may not have its potency strictly limited. The nephrogenous tissue of the proximity of the thirtieth somite may not form any mesonephric tubules in normal development, yet have the potentiality to do so in grafts.

There was not a single indication of metanephros in any of the grafts from donors younger than the fifty-somite-stage (ninety-four hours' incubation). However, in the single graft from a fifty-somite donor, and in the two from fifty-one-somite donors, there was very fine differentiation of metanephros and all of its component parts. Differentiation of kidney in these cases was complete in every way, there being well-defined secreting tubules and glomeruli, as well as collecting tubules and ureter which entered into the cloaca. We have described, in the preceding section of the paper, the histological appearance of the different levels of the ureter. By following the branches of the ureter, we were able to ascertain which were collecting tubules. The tubules which were presumed to be secreting tubules were first identified by their close association with the epithelium of the renal corpuscles. Upon examination, we found that histological differences were present, even at this stage, by which we could determine whether both types were present. The collecting tubules were found to have a simple cuboidal epithelium, which was lower than that of the secreting tubules, which was generally pyramidal or low columnar. The cytoplasm of the collecting tubules was quite clear, in contrast to the granular nature of the cytoplasm of the secreting tubules, which stained considerably darker. In the proximity of the secretory elements there was usually some undifferentiated nephrogenous tissue.

It is striking that complete differentiation of metanephros should appear in the grafts from fifty- and fifty-one-somite donors, while there was no indication of any part of the kidney in grafts of any level from a younger donor. One might expect that the metanephrogenous tissue would differentiate

even though the ureter was not present, but apparently it does not. After the ureter is formed, this tissue might differentiate even though the ureter was absent in the graft, since there is the possibility that the age of the tissue is the factor necessary for differentiation.

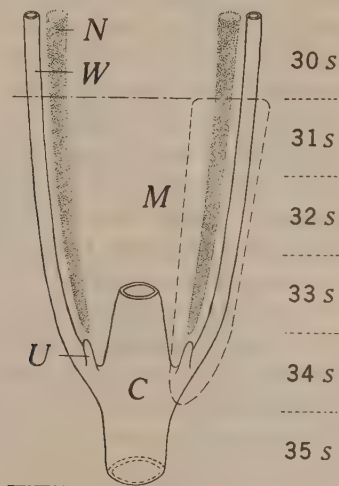


Fig. 2 Diagram of the metanephric primordia as they appear in a fifty-one-somite chick. The isolations which were made, namely, the thirty-one- to thirty-five-somite level and metanephric primordia (*M*), are described in the text. The extent of isolation *M* is indicated by the dash line. *C*, cloaca; *N*, nephrogenous tissue; *U*, ureter; *W*, wolffian duct.

III. Grafts of the isolated metanephric primordia

In order to confirm the results of the experiments just described, a series of grafts were made of the metanephrogenous tissue together with the wolffian duct posterior to the thirtieth somite, from one side only (*M*, fig. 2). In this way, the specific ureter-forming level of the wolffian duct and the nephrogenous tissue were transplanted together. The wolffian duct and the nephrogenous tissue are in very close proximity, and the metanephric diverticulum and nephrogenous tissue are in even closer association immediately after the

former arises. Although it is realized that transplantation of the two primordia separately would constitute a critical experiment, we found that their close association rendered separation impractical.

In order to insure that all of the posterior part of the wolffian duct would be included, a small part of the cloaca was removed with the wolffian duct, as is evidenced by the appearance of cloaca in most of the grafts. This type of isolation is designated as *M*, figure 2. The results of these grafting experiments are given in table 2, the laterality of the isolation being indicated in each case. The isolations were very small and the grafts were correspondingly small. The percentage of successful grafts was very low, only sixteen (35 per cent) grafts were obtained from the forty-six hosts which survived the experimental period.

The results of these grafts confirm those of the preceding experiment in every way. Metanephros did not differentiate in the eleven grafts from donors between the thirty-six-somite stage and the forty-nine-somite stage. In one of the two grafts from fifty-somite donors, and in one of the three grafts from fifty-one somite donors, there was excellently differentiated kidney. A photomicrograph of a section of graft 107-4, which was from a fifty-somite donor, is shown in figure 3. This section is typical of all of the sections of the graft, in that the graft is made up entirely of metanephros, except for a short section of wolffian duct and cloaca. This section is through the level of the graft in which the ureter is branching into smaller tubes which form collecting tubules. The ureter enters the short section of wolffian duct which is in the graft at another point. The nephrogenous tissue apparently collected in masses about the ends of the secondary branches of the ureter and there differentiated into secreting tubules and renal bodies. There is still evidence of undifferentiated nephrogenous tissue accompanying the differentiated tubules. We see, then, that transplantation of the metanephric primordia, at a time when both are definitely indicated and in contact, yields a complete, though minute, kidney.

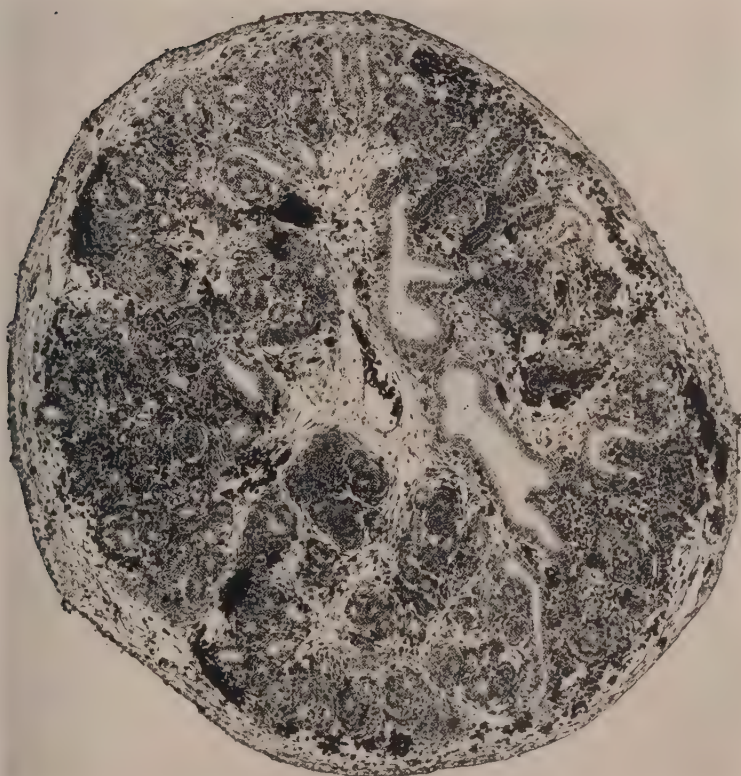


Fig. 3 Photomicrograph of a section through graft 107-4, from an implant of the left metanephric primordia of a fifty-somite embryo. The entire graft is composed of kidney tissue. This section is taken at the level where the ureter is breaking up into collecting tubules. $\times 125$.

DISCUSSION

It is shown by the above experiments that the material of the end-bud and primitive-streak levels of the blastoderm, during end-bud stages, has very little capacity for differentiation of structures other than those of the most generalized type, i.e., gut, smooth and skeletal muscle, cartilage, skin, and feather germs. The end-bud level alone and the primitive-

streak level alone apparently do not have the capacity to produce skeletal muscle and cartilage. The absence of neural tube and notochord offered the most unexpected result, especially in view of the manner in which these axial structures are believed to develop in the posterior part of the normal embryo. The failure of metanephros to differentiate in grafts of these levels is more easily accounted for, in view of the results of the subsequent experiments in which the metanephric primordia were transplanted.

We wish to consider, first, the failure of axial structures to appear in grafts of the end-bud level, and the bearing of this on the problem of the significance of the end bud in the formation of posterior structures of the embryo. The apparent lack of capacity of the end-bud level to form neural tube and notochord is in marked contrast to the capacity of the level containing Hensen's node of presomite and early-somite stages, to form these structures (Hunt, '31). The node level apparently loses its capacity to form neural tube and notochord just before the time of end-bud formation. The node level no longer has the capacity to form neural tube after the disappearance of the rhomboidal sinus (presumptive medullary plate). Wetzel ('29) has shown by his marking experiments that only the floor of the neural tube forms directly from the node material, while the walls and roof are derived from the presumptive medullary-plate material lateral to the node. It seems quite reasonable that all of the neural tube formed up to the time that the end bud first appears and neuropore closes, be laid out as presumptive material in the rhomboidal sinus, since neural tube differentiated in grafts in which a portion of the rhomboidal sinus was included.

If it be true that the presumptive medullary plate for a certain part of the body is laid out in this manner, what, then, is the source of the neural-tube material after the neuropore closes and the rhomboidal sinus has disappeared? Considerable evidence has been presented in favor of the view that the neural tube of the posterior levels forms exclusively

through differentiation of indifferent cells of the end bud and does not arise by the closure of ectodermal neural folds.

This was first noted in the parrot and in the chick by Braun ('82), who recognized two types of neural-tube formation, the first, by a closure of ectodermal folds; the second, in the posterior extremity, from a solid anlage, the 'Medullarstrang,' which becomes hollowed out secondarily. Keibel and Elze ('08) state that in the human embryo the caudal end of the medullary anlage arises not through the growing together and closure of medullary folds, but differentiates with chorda from an indifferent cell mass, the tail bud.

This idea was not developed any further until Holmdahl ('25), from his studies on bird and mammalian embryos, applied it as strong evidence for his theory of primary and secondary development, and Schumacher ('27) described the process very carefully in the chick. Holmdahl believed that all of the neural tube and notochord of the posterior half of the body forms directly from the end bud by a process of canalization of the solid strand. His observations of sections of normal chick and mammalian embryos indicate that there is a gradual transition from the end bud, in which the cells are irregularly arranged in a compact mass; through a stage in which a thinning out in the central part produces a lumen with an epithelium; to the definitive neural tube, an oval, epithelial tube with a dorsoventrally elongated lumen. He conceived of this process being initiated at the time of the closure of the neuropore and the formation of the end bud.

Schumacher's ('27) observations were made in a search for an interpretation for a so-called, teratological condition found by a number of investigators in the caudal levels of the neural tube of bird and mammal embryos. This condition, known as 'multiplicity of neural tubes,' in which there was more than one lumen in the spinal cord, was believed to be an abnormal situation and had been described many times as such, and had even been produced experimentally in the chick by Waelsch ('14) and Gawrilenko ('24). Schumacher has shown, however, that this is not an anomalous situation,

but, in reality, is the normal method of formation of the neural tube in caudal levels. He described and figured (figs. 1 to 6, pp. 84, 86, '27) sections from a normal fifty-seven-hour chick embryo, showing very clearly how differentiation of neural tube and notochord takes place from the end-bud material. During the thinning-out process, there is apparent, in most cases, more than one lumen, which, as differentiation progresses, coalesce to form the single lumen of the definitive neural tube. The notochord is not at first sharply delimited from the medullary strand, but soon separates from it, as neural-tube differentiation progresses.

There seems to be no doubt that this is the usual method of formation of axial structures in the caudal end of the embryo. I have examined a number of series of sections of chick embryos, varying from the twenty-one-somite stage to the fifty-one-somite stage and they all seem to show that these observers were correct in their interpretation that the neural tube and notochord form from the end bud directly, as described.

Since it appears so evident that the neural tube forms from the end bud, it is of interest to observe that this level does not differentiate in the grafts into nervous tissue and notochord. These results indicate that the cells of the end bud are physiologically indifferent. The material may be destined to form these structures, but evidently does not have sufficient organization to give rise to axiate organs. At the time the isolations were made, the end-bud material does not have capacity to self-differentiate structures which are normally traced to it, since only generalized tissues differentiate from it. Holmdahl ('26) and Schumacher ('27) speak of the end bud as being indifferent, apparently in the morphological sense of being histologically undifferentiated, Holmdahl characterizing it as an 'indifferente Zellenmasse,' a 'Sprossungszentrum' for the caudal levels of the embryo. It seems that this interpretation of the end bud is a correct one, since it evidently is a proliferation center for material of the caudal neural tube and notochord, yet is supplying material with a

low grade of determination. That the end bud is a center of marked activity has been demonstrated by Hyman ('27), who employed the method of differential susceptibility to toxic agents. The 'tail bud' was found to be the most active region of the two-day chick. From this, one would expect that the end bud would have a higher degree of determination, but evidently this is not so.

According to Wetzel ('29), the end bud is the same in form and material as the node and anterior part of the streak of earlier stages, namely, the place of formation of notochord, neural tube, and somites. This may be true, yet the end bud differs radically from the node of early stages in that the node is a center of organization possessing a marked degree of determination (Hunt, '31).

Holmdahl ('25) arrived at essentially this same conclusion largely through observational evidence, and formulated from it his theory of two phases in the development of the embryo. He expressed the view that all of the embryo posterior to the midtrunk level forms from indifferent material of the end bud (secondary body development), in contrast to the formation of the head and anterior half of the trunk from determined germ-layer material laid down as the node moves posteriorly along the primitive streak (primary body development).

Holmdahl estimated that the posterior limit of primary development is located about the level of the twenty-seventh or twenty-eighth somite. This estimation was based on his belief that all of the material laid out in front of the end bud at the time of its formation, is the product of primary development. Since the end bud is formed at the twenty-one-to twenty-three-somite stage, and since there is segmental plate at this time capable of forming five or six somites, if no elongation occurs, Holmdahl has estimated that about twenty-seven or twenty-eight somites are formed during primary development. Holmdahl performed a set of experiments which entailed production of a lesion by electrocautery at the posterior end of the differentiated embryonic axis, just in front of the end bud, of a forty-eight-hour chick. Two days

later, the lesion was found in the trunk region, just behind the wing buds. He interpreted this to mean that the level behind the lesion was formed largely from end-bud material, and that the boundary between the two developmental phases is in the lumbosacral region.

Gräper ('27) has criticized this experiment on the ground that the embryos were not vitally stained before the lesion was made, so that there was no certainty as to where the marks were made. Gräper was of the opinion that the end bud does not play such an important part in body formation, but that a major portion of the caudal levels arises by elongation and growth of material in front of the end bud.

The posterior boundary established by Holmdahl as the extent of primary development from determined material, is in very close agreement with the posterior extent of structures differentiated in grafts of the node of presomite and early-somite stages (Hunt, '31) and in grafts of whole blastoderms of the same stages (Willier and Rawles, '31). Mesonephros was the most posteriorly situated organ found in grafts of the node level or whole blastoderms of presomite stages. Mesonephros is found between the fifteenth and thirtieth somites, but we have no evidence that all levels of the mesonephros were represented in these grafts. We see no very good reason, on the other hand, why the posterior levels of the mesonephros were not present, except that gonad, which is normally in close association with mesonephros in the region between the twenty-first and twenty-sixth somites, was not present in these grafts. Gonads did appear, however, in grafts of whole blastoderms of early-somite stages, so it is believed that all levels of the embryo back to about the thirtieth somite are determined by the time somites begin to appear. The single criterion of a level caudal to the thirtieth somite, namely, metanephros, is missing from these grafts. So we see that these results agree fairly well with Holmdahl's theory that structures back to about the twenty-eighth somite are formed during primary development from determined material.

The transplants of the end-bud level of the embryo, described above, while confirming the idea that the end bud is made up of undetermined material, do not throw much light on the question of whether the end bud actually contributes the material for the formation of caudal parts, which Holmdahl has postulated as the second phase of the developmental process. We are convinced that he is correct in his interpretation that neural tube, notochord, somites, etc., of caudal levels are formed from the indifferent cells of the end bud. We have already discussed the evidence that neural tube and notochord are formed from the end bud. It appears that Holmdahl's conception of two phases in the development of the embryo is justified, since there is such a marked difference in the time of determination of the structures of the anterior and posterior levels of the body. Neural tube, notochord, and metanephros are not determined in the caudal levels until the time that the rudiments of these organs are about to appear, while the potencies of structures of anterior levels are determined long before the time of histological differentiation, as evidence by the determination of mesonephros in the primitive-streak stage. That the material proliferated by the end bud actually acquires specific potency for neural tube and notochord is indicated by four grafts, not previously mentioned, of the tail bud, which included besides the end bud and remnant of the primitive-streak mass beneath it, a short section of neural tube and notochord which had formed from end bud material. These grafts—one (N-5) from a twenty-nine-somite donor, two (J-1, J-14) from thirty-one-somite donors, and one (O-1) from a thirty-three-somite chick—differentiated into neural tube, notochord, gut, smooth muscle, cartilage, skeletal muscle, skin, and feather germs. This result indicates that as soon as the rudiments of the neural tube and notochord are formed, the potencies become fixed.

It is of interest in this connection to point out that Bijtel ('31) has recently contributed some evidence in connection with this problem in urodeles and anurans. As a result of

his vital-staining experiments, he is of the opinion that the organs of the tail of these larvae do not form from indifferent cell material of the tail bud, but that the neural tube and notochord are formed by elongation of the anlagen of these organs following the medullary-plate stage.

We wish to consider, now, the problem of the differentiation of the metanephros. We have shown that metanephros does not form in grafts taken from donors younger than the fifty-somite stage. Willier ('30, p. 208) had previously obtained metanephros in grafts of the entire wolffian ridge of five-day embryos. That metanephros does not form earlier than just indicated was found to be true in grafts of the level of the embryo in which the metanephric primordia arise, and in grafts of the metanephrogenous tissue together with the section of the wolffian duct that gives rise to the ureter.

Since the fifty- or fifty-one-somite stage is the earliest stage in which both primordia (metanephrogenous tissue and metanephric diverticulum) are definitely indicated, we seem justified in the conclusion that both primordia must be present in the graft at the time of its isolation. While not positive that the metanephric diverticulum was present in these grafts at the time the isolation was made, we infer so from studies of preparations of normal embryos which showed definitely that the diverticulum is present in fifty- or fifty-one-somite embryos.

It seems that the wolffian duct apparently does not have the capacity to form a ureter in grafts. We may rule out the possibility that ureter formation is dependent on nephrogenous tissue, since nephrogenous tissue is present in all of the grafts from stages later than thirty-five somites in exactly the same relationship to the wolffian duct as it bears in normal development, yet ureter does not arise in these grafts. In many of the grafts in which metanephros did not occur, the specific ureter-forming level of the wolffian duct was present, yet the ureter seems to have been inhibited from forming. Boyden ('28) has presented evidence that the wolffian duct must enter the cloaca before it will form a ureter, but in all

of these grafts from embryos with over forty somites, the wolffian duct had entered the cloaca, yet no ureter develops. Lillie ('04) destroyed varying amounts of the posterior end of twenty-nine-somite chick embryos by cauterization, and found that kidneys and ureters were absent in the defective embryos which resulted. Since the metanephric level of the wolffian duct was destroyed by the operation, he postulated that ureter formation is either limited to a short region of the wolffian duct or is dependent on a certain amount of kind of development of the allantois or cloaca.

The fact that nephrogenous tissue will not differentiate until the ureter appears, suggests that differentiation of the former is dependent on the formation of the ureter. We noted before that in every case in which differentiation of metanephros occurred, all of the component parts were present. The presumptive metanephrogenous tissue was present in all of the grafts from stages over the thirty-five-somite stage, yet did not differentiate independently of the ureter. There was no indication of independent acquisition of potencies of secreting tubules and renal corpuscles, unless the nephrogenous tissue acquired these specific potencies simultaneously with the appearance of the ureter—a possibility which is not very likely. The most critical experiment to test the capacity of nephrogenous tissue to differentiate independently, would be to separate the two primordia just after the ureter had appeared, but, as we have pointed out previously, their close approximation makes this impractical.

That the differentiation of the nephrogenous tissue is dependent on the presence of the metanephric diverticulum, is in agreement with the view expressed by Boyden ('28) as a result of his experiments in which he prevented the posterior growth of the wolffian duct in the chick. As the wolffian ducts did not enter the cloaca in these cases, presumably, the ureters failed to appear, and no secreting tubules and renal bodies were formed. There was a concentration of nephrogenous tissue medial to the posterior cardinal veins, but this blastema never differentiated into secreting tubules and renal cor-

puscles. Boyden ('32) also reports a 10-mm. human embryo in which the left wolffian duct failed to descend below the level of the stomach, resulting in the absence of the left ureter. The left metanephric blastema was fused to the right kidney, but did not differentiate into tubules.

Hoadley ('26), on the contrary, presented evidence that in the case of mesonephros, the secreting elements were determined first and appeared in grafts from younger donors than did the collecting tubules and wolffian duct. This would indicate independent origin and differentiation of the secreting and collecting elements of the mesonephros.

As to the nature of the action of ureter on the nephrogenous tissue in the metanephros, these experiments offer no suggestion.

SUMMARY

1. The end-bud and primitive-streak levels of the embryonic axis of end-bud stages of the chick embryo have the capacity to form only generalized, non-axiate structures in chorio-allantoic grafts. Neural tube, notochord, and metanephros do not differentiate in these grafts.

2. Evidence is presented to show that neural tube and notochord of caudal levels normally form directly from the end bud, the former by the canalization of a solid strand instead of by closure of ectodermal neural folds. Even though these structures are traced to the end bud, the grafting experiments show that the latter does not have specific potency for neural-tube and notochord formation.

3. The end-bud level is, therefore, shown to be a physiologically indifferent cell mass, in contrast to Hensen's node level of presomite stages, which has been shown to have capacity for differentiation of almost all structures of the anterior half of the embryo.

4. Structures of caudal levels—neural tube, notochord, and metanephros—are not determined until the material is actually incorporated in the rudiments of these organs, while structures of the body anterior to approximately the twenty-sixth to thirtieth somite level are determined as early as the

primitive-streak and head-process stages. Since there is such a marked difference in time of determination of structures of anterior and caudal levels, Holmdahl's theory of two phases in the development of the embryo seems justified.

5. Grafts of the prospective metanephric level (thirty-one to thirty-five somites, inclusive), and grafts of the isolated metanephric primordia from fifty- and fifty-one-somite embryos, differentiated into kidney which was complete in every respect, while grafts of the same types from younger donors did not form metanephros in any case. Since this is the earliest stage in which the ureter is present and in contact with the metanephrogenous tissue, it is believed that this condition must exist before metanephros will differentiate.

6. As the metanephrogenous tissue did not in any case differentiate independently of the ureter, even though present in many grafts in which the metanephric diverticulum was absent, it is suggested that the differentiation of the metanephric blastema is dependent on ureter formation.

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VARIATIONS IN THE ARRANGEMENT OF THE BRANCHES ARISING FROM THE AORTIC ARCH IN AMERICAN WHITES AND NEGROES¹

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ONE FIGURE

In 1895, Sir Arthur Keith wrote: "The cynomorphous arrangement of the primary branches of the aortic arch occurs as a variety in the negroid races with thrice the frequency found in white races." The writers' search of the literature has revealed no similar conclusion. It is inferred that by the term 'cynomorphous arrangement' Keith refers to that arrangement in which there is common origin from the aortic arch of innominate and left common carotid arteries, with separate left subclavian artery.

During the school year 1930-1931, one of the routine research problems assigned to two students of the class in gross anatomy of Washington University School of Medicine was that of the variation in branching of the aortic arch. The problem was repeated by two members of the class of 1931-1932. Through the kindness of Prof. D. M. Schoemaker, of the Department of Anatomy of St. Louis University, fifty-one more cadavers were examined, and, in the School of Dentistry of Washington University, nine others. In all, 159 specimens were investigated, distributed as to race and sex as indicated in table 1.

The classification adopted for the types of arrangement of the branches of the aortic arch was that of Adachi ('28). Three varieties not given in his list were added. Figure 1 depicts all these types diagrammatically.

¹ The author wishes to acknowledge aid given to this investigation by the Rockefeller Foundation.

TABLE 1
Incidence of anomalies of branching of the aortic arch

TYPE	A	B	C	D	E	F	G	H	I	BE	TOTAL
This study:											
White males	54	14	2				1			1	72
	75.0%	19.4%	2.8%				1.4%			1.4%	
Negro males	26	28						1	1		56
	46.4%	50.0%						1.8%	1.8%		
White females	5	2						1			8
Negro females	14	8						1			23
	60.9%	34.8%						4.3%			
Thompson, 1893 ¹ :											
English	412	51	27	2			4				500
	82.4%	10.2%	5.4%	0.4%			0.8%				
Adachi, 1928	430	56	22	3	3	1	1				516
Japanese	83.3%	10.9%	4.3%	0.6%	0.6%	0.2%	0.2%				

¹ In addition to the cases listed, Thompson reported two, in which the thyroidea ima artery arose from the aortic arch; one case similar to type H, except that the left vertebral artery arose from the arch between the bicarotid and left subclavian arteries; one case in which each common carotid, each subclavian, and the right vertebral artery (five in all) arose from the aortic arch.

Observation and notation of the type of branching of the aortic arch may be made in two ways, both of which were employed to obtain the data here presented. It is sufficient in many cases to examine the branches without opening into the lumen of the arch. However, it was found desirable as a check method and sometimes necessary (especially in the cases of very short common stems originating from the arch) to expose to view the lumen of the arch by slitting it along its concavity. By this maneuver, the lumens of origin of the

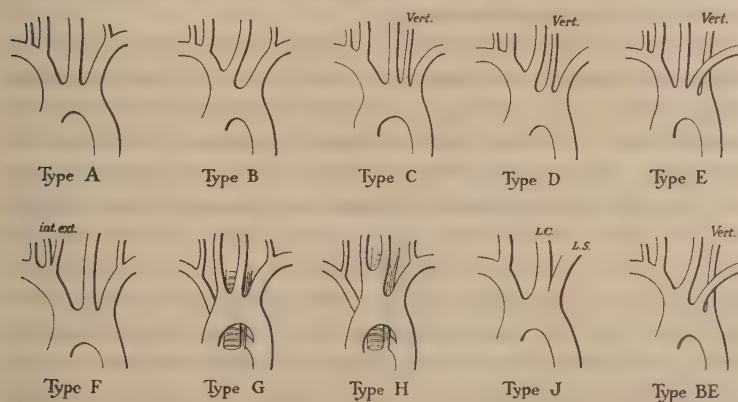


Fig. 1 Types of variations in the arrangement of the branches arising from the aortic arch (based on Adachi's classification).

branches are clearly seen. The writers believe that had not this latter method of examination been used, several cases of very short common stems of origin of innominate and left common carotid arteries (type B) would have been designated as cases in which the two arteries named have separate origin (type A). The use of the second method no doubt accounts for the comparatively high incidence of type B in the whites of this study as compared with that of the whites from other sources. It should be noted, however, that all cases designated as belonging to type B showed partitions between the innominate and left common carotid arteries which were

definitely within the lumens of the common stems and which were definitely superior to the arc of the convexity of the aortic arch.

Table 1 shows the absolute and relative incidence of the types of branching of the aortic arch in American whites and negroes of both sexes. The number of observations for females of either race is small. Adachi ('28) found no significant sex difference in incidence of type A. The writers believe that the same is true for their material, but until larger samples are obtained for each group, they prefer to treat each group separately.

The preponderance of type A in all white males and the practically equal incidence of types A and B in American negro males are striking. Keith's statement that type B "occurs as a variety in the negroid races with thrice the frequency found in the white races" is almost literally true for the relative incidence of American negroes and whites; i.e., 2.6 times as frequent in the American negroes of this series. The findings here presented are not in agreement with those of De Garis ('25). One of his forty-five white males showed the thyroidea ima artery originating from the arch, one belonged to type C, and the remainder to type A; in seventy-eight American negro males there were found seventy type-A patterns, two of type B, one each of types C and G, one case of a bicarotid artery with separate right and left subclavians, and three cases of origin of the thyroidea ima artery from the arch. One white and one negro female each manifested the last designated anomaly, but the other eight in each group were all of type A. Loth ('12) reports fifty-one cases of dissection of the aortic arch of negroes conducted by himself or taken from the literature. Twenty-four, or 78 per cent, are described as normal, three are unclassifiable (but two of these may belong to type B), one to type D, and three to type G.

In the writers' material are recorded four cases of origin of the right subclavian artery from the thoracic aorta (types G and H). No sexual nor racial difference in incidence is

indicated. In each case, the anomalous branch passed on its way to the right upper extremity posterior to both trachea and oesophagus. In this particular series of these anomalous cases it is interesting to note that three of the four were characterized by the presence of a second anomaly—that of bicarotid artery (type H rather than type G).

The appearance of the left vertebral artery as a branch of the aortic arch is confined entirely to whites in the three cases found. In two of the cases, the supernumerary branch arose between the left carotid and left subclavian arteries (type C); in the third, it originated from the arch to the left of the origin of the left subclavian, and in this case also, the innominate and left common carotid arteries had a common origin (type BE).

The one remaining anomalous case is that of two innominates arising from the arch (type J). The length of the left innominate in this case was approximately 1 cm.

CONCLUSION

The data here presented for examination and discussion indicate agreement with Keith's dictum of 1895, that the common origin of the innominate and left common carotid arteries from the aortic arch is much more frequent in negroids than in whites.

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THE EFFECT OF HYPOPHYSECTOMY ON THE SURVIVAL OF SPERMATOOZOA IN THE MALE RAT¹

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TWO FIGURES

Studies upon the effect of hypophysectomy on the survival of mature spermatozoa have not been reported, although a number of papers have appeared on the influence of castration on sperm motility and survival. The effects of the two types of operations are similar as regards certain features and might be expected to give a similar survival period of the sperm. The accessory organs after hypophysectomy undergo about the same degree of atrophy as after castration, and this atrophy is presumably the immediate cause for the decrease in the survival period of sperm which has been shown by a number of investigators.

After hypophysectomy, however, there are certain effects which are not present after castration. Some of these might tend to further decrease the survival period of the sperm, whereas others might have the opposite effect. There is a profound systemic effect from hypophysectomy. Rats show varying degrees of cachexia, and certain organs which are not affected by castration undergo atrophy. The body temperature is lowered. Although an increased temperature has a deleterious effect upon sperm survival, the effect of a sub-

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normal temperature has not been reported. There is a possibility that the pituitary sex hormone may influence directly the mature sperm, although it has only an indirect effect upon the accessories. The amount of this hormone is increased in the circulation of rats by castration as evidenced by parabiosis experiments. After hypophysectomy it is absent.

Thus there are a number of conditions which are widely different in the castrated and the hypophysectomized animal. It consequently seemed worth while to investigate the period of survival of mature sperm in hypophysectomized rats and to compare it with that of castrated animals.

METHODS

Before attempting to make determinations on the survival of spermatozoa in the hypophysectomized rat, it seemed advisable to perform a series of operations conforming to those already reported in the literature. In this way a satisfactory technic was assured and material obtained for a comparison of the vitality of our colony of animals with those used by other investigators. Animals which were normal but were not litter mates were used in the preliminary work. These experiments demonstrated that, after bilateral operations, one side of the reproductive tract could be removed aseptically without affecting the survival of spermatozoa in the other side. By employing this procedure, motility determinations were made on each animal after two different postoperative intervals. However, if no motile sperm were found at the first examination, the animal was killed and the procedure repeated on the other side. The advantages of this technic are obvious when the difficulties of hypophysectomy are recalled.

In most of the experiments reported here, a series of litter mates was used. In each litter one animal was castrated, another bilaterally vasa efferentia ligated, and a third hypophysectomized. Although a number of unilaterally castrated animals have also been used in these experiments, in order to

compare the results obtained with those reported in the literature, this operation was not included in the series of litter mates. Bilateral ligation of the vasa efferentia (ductuli efferentes) was largely substituted for it, since this procedure gives similar results and has the distinct advantage of allowing motility readings to be made on both sides of the reproductive system of the animal. All operations on a given litter were done on the same day; the litter mates were kept in the same cage under identical conditions, and, with a few exceptions, sperm motility readings were made on all three animals of a set after the same postoperative interval.

All operations were done aseptically and under ether anaesthesia. The hyophysectomies were done according to the procedure described by Dr. P. E. Smith in 1930. The total removal of the anterior and posterior pituitary was confirmed by serial sections.

In the operations upon the testis the scrotal approach was used entirely. This approach requires a longer procedure than that using the abdominal route, but eliminated the uncertainty as to whether the epididymis had returned to the scrotum, which was highly desirable in these experiments. A brief description of the operative technic may be given. The hair was shaved from the surface of the scrotum, which was then sterilized. A median incision was made in the skin, exposing the testes lying in their protecting sheaths. A hole was made in the tunica vaginalis, through which the testis and epididymis were retracted. In the bilateral castrations the internal spermatic vessels and the vasa efferentia were ligated with a single ligature and the testis removed, leaving the entire epididymis unharmed. The tunica vaginalis was sutured with silk and the procedure repeated for the other side. The skin wound was then closed with suture clips. The technic is similar for unilateral castration or, as it is sometimes called, epididymal isolation, only one side, however, being opened and a single testis removed. In the vasa-efferentia ligations the vasa efferentia were carefully dissected away from the internal spermatic vessels and

ligated in two places. The tubules were then severed between the two ligatures. This part of the operation was done under a dissecting binocular. The blood vessels to the testis were left quite unharmed. The completeness of the ligation was checked under the binocular when the organs were removed for motility examinations. The first side to be examined was removed by an aseptic operation, the animal being killed when the second examination was to be made. In all of the non-castrate operations the testes were fixed after the motility determinations and the sections studied for degenerative changes.

At the time of operation all animals were measured and weighed. The hypophysectomized individuals subsequently were weighed every day as a check on the totality of the operation. The other animals were weighed twice a week.

The motility examinations were made as follows: the extirpated side of the reproductive tract was placed under a half Petri dish, on filter-paper moistened with physiological saline. A portion of the ablated tract was snipped off and teased in a small quantity of a physiological solution on a flat-bottomed depression slide. All determinations were made under the low power of the compound microscope at room temperature under uniform conditions.

To avoid transferring motile sperm from one level to another, the instruments were carefully cleansed after the examination of each level.

A number of tests were made with various solutions—Ringer's, Locke's, Baker's, Tyrode's, and normal saline. After trying portions of the same samples of sperm in each of these fluids, Tyrode's was selected.

In the castrated animals sperm from the head of the epididymis, tail of the epididymis, and ductus deferens were examined. In the remainder of the animals the testis also was examined. The quantitative scheme developed by Moore ('28) has been used for recording the degree of motility. In this system the maximum motility exhibited by sperm is designated ****. When there are a few inactive sperm, the ma-

jority still being quite active, the motility is ***. Likewise, when there are about 50 per cent of the sperm still somewhat motile, the reading is **. When there are only a few feebly active sperm, even one or two on a slide, the reading is *. Finally, if there is no motion in any of the sperm, the reading is 0. Following this system the normal motility of sperm from the ductus deferens is ****; from the tail of the epididymis ****; from the head of the epididymis ***; and from the testis *. If the first examination of a given region was negative, another specimen was examined before the final result was recorded. The readings in the series of animals were made at intervals of three days, which was considered to be the shortest significant difference in survival time.

SURVIVAL OF SPERMATOZOA IN NON-HYPOPHYSECTOMIZED ANIMALS

The non-hypophysectomized animals were divided into three groups, one of which was bilaterally castrated, another unilaterally castrated, and the third bilaterally vasa-efferentia ligated. In addition to those, in order to make the comparison with hypophysectomized animals as significant as possible, litter mates were used in another series of examinations which covered the period from three to twenty-seven days after operation, one animal of each litter being bilaterally castrated, another bilaterally vasa-efferentia ligated, and a third hypophysectomized. To facilitate the presentation of the data, all of the results of the examination of non-hypophysectomized individuals have been included in one table (table 1), and of the hypophysectomized animals in another (table 2). In order that the reader may know which individuals are litter mates, superscripts have been added to the colony numbers.

Bilateral castration

The data from the bilaterally castrated animals are given in section A of table 1. The results differ slightly from previous reports on the survival of spermatozoa after castra-

TABLE 1
Survival of spermatozoa in non-hypophysectomized rats

ANIMAL Colony number ¹	OPERATION		EXAMINATION			MOTILITY			
	Weight	Date	Days after operation	Weight	Side	Epididymis		Ductus deferens	Testis
						Head	Tail		
A. Bilateral castration									
BH5120 ¹	208	3/23	6	197	Left	*	****	***	
BH5121 ²	215	3/26	9	226	Right	*	***	*	
BH5120 ¹	208	3/29	12	214	Right	0	**	**	
BH5173 ³	247	3/29	12	254	Left	0	**	**	
BH5121 ²	215	4/1	15	236	Left	0	**	**	
BH5173 ³	247	4/1	15	251	Right	0	*	0	
GH5030 ⁴	215	5/1	15	245	Left	0	0	0	
GH5030 ⁴	215	5/1	15	245	Right	0	*	*	
GH5182	237	4/15	18	243	Left	0	*	*	
GH5092 ⁵	255	4/15	18	260	Left	0	0	0	
GH5092 ⁵	255	4/15	18	260	Right	0	*	*	
W5648	294	6/25	21	289	Right	0	*	*	
W5648	294	6/25	21	289	Left	0	*	*	
BH5653	290	6/25	21	284	Right	0	*	*	
GH5387 ⁶	336	5/10	24	376	Right	0	0	0	
GH5387 ⁶	336	5/10	24	376	Left	0	0	0	
W5286 ⁷	289	5/13	27	313	Right	0	0	0	
W5386 ⁷	289	5/13	27	313	Left	0	0	0	
B. Unilateral castration									
GH5091	267	4/15	18	255	Left	0	***	***	
GH5093	238	4/21	24	263	Left	0	**	**	
GH5181	244	4/27	30	303	Left	0	**	**	
W5835	294	8/3	33	320	Left	0	*	0	
BH5010	299	8/6	36	328	Left	0	*	*	
B5652	264	7/13	39	306	Left	0	0	0	
W5650	231	7/16	42	246	Left	0	*	0	
W5651	270	7/19	45	316	Left	0	*	0	
W5649	250	7/22	48	297	Left	0	0	0	
GH4978	302	8/18	48	348	Left	0	0	0	
C. Bilateral vasa-efferentia ligation									
GH5123 ¹	231	3/23	6	245	Left	**	****	***	*
GH5125 ²	196	3/26	9	209	Left	0	***	0*	*
BH5175 ³	228	3/29	12	230	Left	0	***	***	0
GH5125 ²	196	4/1	15	225	Right	0	***	***	*
BH5175 ³	228	4/1	15	229	Right	*	***	***	*
GH5029 ⁴	196	5/4	18	240	Left	0	**	**	0
BH5088 ⁵	252	4/15	18	243	Right	0	*	0	*
GH5123 ¹	231	4/7	21	280	Right	0	*	0	0
BH5088 ⁵	252	4/18	21	249	Left	*	**	**	0
GH5389 ⁶	320	5/10	24	348	Left	0	**	0	0
W5287 ⁷	252	5/13	27	314	Left	0	**	*	0
G5025 ⁴	223	5/16	30	326	Left	0	*	0	0
GH5029 ⁴	196	5/19	33	262	Right	0	*	0	0
G5025 ⁴	223	5/22	36	321	Right	0	*	*	*
W5287 ⁷	252	5/25	39	319	Right	0	*	0	0
GH5389 ⁶	320	5/28	42	346	Right	0	*	0	0
BH5172	204	5/4	45	236	Left	0	0	0	0
BH5172	204	5/4	45	236	Right	0	0	0	0

¹ All animals with the same superscript are litter mates which were kept under identical conditions, and with a few exceptions, examined after the same post-operative period. See table 2.

TABLE 2

Survival of spermatozoa in hypophysectomized rats

ANIMAL	OPERATION		EXAMINATION			MOTILITY				
	Colony number ¹	Weight	Date	Days after operation	Weight	Side	Epididymis		Ductus deferens	Testis
							Head	Tail		
A. Hypophysectomy										
GH5122 ¹	244	3/20	3	218	Right	***	****	****	*	
GH5122 ¹	244	3/23	6	204	Left	**	***	***	*	
GH5124 ²	234	3/23	6	200	Right	***	***	***	*	
BH6281	253	2/2	9	221	Left	*	***	***	*	
GH5124 ²	234	3/26	9	198	Left	**	***	***	*	
B6240	218	2/2	9	180	Left	**	***	***	*	
BH5176 ³	256	3/29	12	226	Left	*	***	*	*	
BH6281	253	2/5	12	210	Right	*	**	**	*	
BH5176 ³	256	4/1	15	219	Right	0	**		0	
B6240	218	2/8	15	176	Right	0	*	**	*	
G5028 ⁴	229	5/1	15	219	Left	0	*	**	*	
G5028 ⁴	229	5/4	18	204	Right	0	*	*	*	
W5284 ⁷	337	5/4	18	266	Left	0	**	**	0	
GH5996	255	7/13	18	240	Left	0	*	*	0	
GH6994	258	7/13	18	218	Left	0	*	*	0	
BH5089 ⁵	275	4/15	18	225	Left	0	*	*	*	
BH6303	286	2/28	21	194	Left	0	*	0	0	
BH5089 ⁵	275	4/18	21	223	Right	0	0	*	0	
W5832	228	7/16	21	213	Left	0	*	0	0	
W5833	262	7/16	21	231	Left	0	*	*	0	
W5832	228	7/19	24	206	Right	0	0	0	0	
W5833	262	7/19	24	226	Right	0	0	0	0	
GH5994	258	7/19	24	212	Right	0	0	0	0	
GH5996	255	7/19	24	234	Right	0	0	0	0	
BH5386 ⁶	341	5/10	24	302	Left	0	0	0	0	
BH5386 ⁶	341	5/10	24	302	Right	0	0	0	0	
BH6303	286	3/5	27	186	Right	0	0	0	0	
W5285 ⁷	304	5/13	27	240	Left	0	0	0	0	
W5285 ⁷	304	5/13	27	240	Right	0	0	0	0	

B. Hypophysectomy and bilateral vasa-efferentia ligation

GH4941	319	5/27	15	260	Left	0	*	*	0
GH4941	319	5/30	18	261	Right	0	*	*	0
GH4942	349	6/2	21	303	Left	0	*	*	0
GH4942	349	6/5	24	294	Right	0	0	0	0
GH5976	366	6/5	24	296	Left	0	0	0	0
GH5976	366	6/5	24	296	Right	0	0	0	0

C. Hypophysectomy and bilateral castration

GH5992	263	7/13	18	227	Left	0	*	*	
GH5993	266	7/13	18	258	Left	0	0	*	
GH5993	266	7/16	21	251	Right	0	*	*	
W5836	232	7/16	21	203	Left	0	0	*	
W5836	232	7/19	24	193	Right	0	0	0	
GH5992	263	7/19	24	216	Right	0	0	0	

¹ All animals with the same superscript are litter mates which were kept under identical conditions, and with a few exceptions, examined after the same post-operative period. See table 1.

tion. Moore ('28) found motile sperm in only one of five rats examined eighteen days after bilateral epididymal isolation (bilateral castration). Likewise he found motility in only two of seven cases examined at sixteen days after this operation, and secured negative results in four out of fifteen animals examined from four to fourteen days after operation. Similarly, Yochem ('30), who extended his observations to the head of the epididymis and ductus deferens, in addition to the tail of the epididymis, found motion in the head of the epididymis for eight days and in one out of eight cases examined at ten days after operation, for twelve days in the ductus deferens, and in the tail of the epididymis in only one case of the six he examined after a sixteen-day postoperative period. He did not prolong his observations beyond this time. In our experiments motility was found for twenty-one days in the tail of the epididymis, for twenty-one days in the ductus deferens, and for nine days in the head of the epididymis.³

The animals of our colony have been inbred for about eighteen years. They were well nourished and in perfect condition, and were about seven months of age—conditions which would favor maximum vitality of their spermatozoa. This may explain the definitely longer survival of the spermatozoa in our animals as well as the greater uniformity in the data.

Unilateral castrations

In order to check further our results against the reports in the literature, a series of unilaterally castrated animals was examined. Benoît ('26) showed that the spermatozoa in the isolated epididymis of animals with one intact testis survived for a much longer time than those in other animals with both testes removed. Moore ('28) found the motility of sperm from the tail of the epididymis to decrease sharply in his unilaterally castrated rats between eighteen and twenty days

³ This expression is used for the sake of brevity throughout the paper and means a capacity for exhibiting motility when placed in Tyrode's solution.

after operation, but continued to find motility up to thirty-one days. In one case at thirty-four days and one out of five examined at forty-one days after operation he also found feeble motion. In addition, he also found this longer survival of epididymal sperm when the remaining testis of unilaterally castrated animals was placed in the abdomen (experimental cryptorchidism), the isolated epididymis as before remaining in the scrotum. The results of our examinations are given in section B of table 1. Here again it was found that the sperm lived longer than is reported by the other investigators. Motion persisted for thirty-six days in the ductus deferens and for forty-five days in the tail of the epididymis.

Vasa efferentia ligation

Unilaterally castrated animals were suitable for determining the maximum survival of spermatozoa in the rats of our colony as compared with that reported by other investigators. However, a technic was desired which would give similar results, but which, in addition, would afford two motility examinations from each animal. Since the germinal tissue of the testis does not have to be actively proliferating to cause normal survival of sperm,⁴ it seemed probable that the ligation of the vasa efferentia, although causing degeneration of the seminiferous tubules, would nevertheless result in a longer survival of sperm than occurs in the castrated animal. Therefore, the vasa efferentia of several animals were ligated in two places and severed between the ligations. When these animals were examined, it was found that the sperm survived just as long in these individuals as in animals with one undisturbed testis. For this reason we have used this operation and bilateral castration as controls for the hypophysectomized individuals instead of unilateral castration with which we could have obtained only one motility determination from each animal. The results from these operations have been

⁴ Moore ('28), after isolating the testis and elevating it into the abdominal cavity, states that if the epididymis remains in the scrotum the sperm survive just as long as if the testis had been actively carrying on spermatogenesis.

included in section C of table 1. It will be noted that, whereas motility was lost comparatively early in the head of the epididymis, it persisted in the tail of the epididymis for at least forty-two days. The motility in the ductus deferens varies somewhat in different animals—a few having large numbers of non-motile sperm after comparatively short postoperative intervals, other having motile sperm up to thirty-six days.⁵ The motion in the testis, while generally found in the normal rat testis and after short postoperative periods, is so slight as to be readily overlooked after the longer periods.

From the sections which were made of the testes of these animals we have been able to confirm the findings of Van Wagenen ('25), Oslund ('26), and Cunningham ('28) concerning the degeneration of the testis after vasa-efferentia ligation.⁶ The results here are quite different from those after ductus-deferens ligation, after which there is little or no degeneration of the testis. The amount of degeneration after vasa-efferentia ligation varies with the different animals, depending on the length of time after ligation. The seminiferous tubules show definite signs of necrosis at the earliest time examined—six days after operation. After twelve days there are numerous giant cells (symplasma of the German workers), formed from coalesced degenerating cells, lying in the tubules. Spermatogenesis has practically

⁵ It was found in all the types of operations that the readings from the tail of the epididymis were more uniform than those from other parts of the reproductive tract, and survival there was also generally longer, particularly after those procedures which allowed maximum survival (unilateral castration and bilateral vasa-efferentia ligation).

⁶ Moore ('31) found in guinea-pigs, in which a ligature had been passed around the head of the epididymis six months previously, that 60 to 80 per cent of the tubules in a cross section of the testis were normal. If the conditions in the guinea-pig are similar to those in the rat, the failure of the tubules to degenerate must be due either to an incomplete ligation (the ligature being too loose to completely occlude the passages) or to the distance from the testis at which the ligature was applied (one-third of the head of the epididymis remained intact). It is possible that the tubules did degenerate and that regeneration took place after six months in these animals. None were examined earlier to determine this point. The longest period after vasa-efferentia ligation was far advanced and no indications of regeneration were found (Oslund, '26).

disappeared at this period. From fifteen to twenty-four days after ligation the degeneration is progressive until only spermatogonia and Sertoli cells remain in the tubules, and after six weeks these cells are even further decreased in number. After the shorter periods the tubules increase somewhat in diameter, but in the later stages there is a marked decrease in size, the intertubular spaces in the sections being filled with interstitial tissue and a granular precipitate of lymph. Nevertheless, at this time there is an apparent hyperplasia of the interstitial cells, but no quantitative studies⁷ (Bascom, '25), which would be necessary to demonstrate this point, were made. Although the testis remains readily palpable for at least six weeks, the gross size of the organ seems somewhat decreased at this time. It is obvious that complete ligation of the vasa efferentia is necessary not only to secure the ultimate degeneration of the testis, but even more so to determine the survival of previously formed spermatozoa. For these reasons the operations have been done under the dissecting binocular and closely checked at the time of motility examination for any accessory ducts or incomplete ligations.

SURVIVAL OF SPERMATOOZOA IN HYPOPHYSECTOMIZED ANIMALS

Hypophysectomy

Having determined the survival time of spermatozoa in our animals, it was then possible to make comparisons with survival in hypophysectomized rats. To make the results more comparable, a series of operations was performed on sets of three litter mates, as previously mentioned, one being hypophysectomized and the other two bilaterally castrated and bilaterally vasa-efferentia ligated, respectively. Since in the ligated animals the sperm survived for approximately the same time as in normal animals (that is, animals with an intact functional testis, as is the case after unilateral castration) and sperm survive for a much shorter time in animals deprived of their testes, these individuals served

⁷ Weights of these testes were not obtained, since parts of the testes were used for motility examinations as soon as possible after removal.

as controls for the hypophysectomized ones. The results of the examinations, which cover a period of three to twenty-seven days after hypophysectomy, are given in section A of table 2.

These examinations show that there is a definite decline in the degree of motility of sperm from the tail of the epididymis at about fifteen days after hypophysectomy. From this time on slight motion was found until twenty-one days, after which it disappeared altogether. The drop in motility occurs after about twelve days in the ductus deferens, but here also motile sperm were found for twenty-one days. The

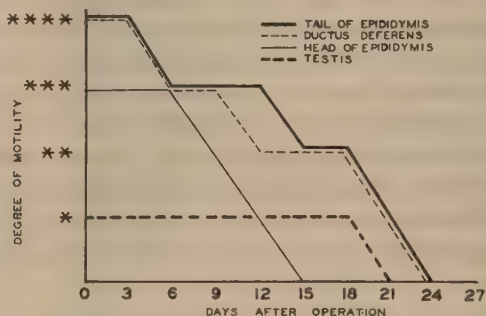


Fig. 1 Graph showing the maximum degree of motility exhibited by spermatozoa removed from the different segments of the reproductive tract of hypophysectomized rats after various postoperative intervals.

few remaining sperm in the head of the epididymis lose their motility after twelve days. It has already been pointed out that motility in the testis of the rat is very slight (in most other species it is imperceptible). There is always, however, a slight bending motion in a number of sperm from the normal testis. This slight motion was detected for eighteen days following this operation, after which it disappeared. Figure 1 shows graphically the degree of motility exhibited by sperm from each of the segments of the reproductive tract for the various days after hypophysectomy.

The contrast between these results and those after bilateral vasa-efferentia ligation is striking. Motile sperm were found

after the latter operation for forty-two days in the tail of the epididymis, for thirty-six days in the ductus deferens, for twenty-one days in the head of the epididymis, and for eighteen days in the testis (in one case motility was observed after thirty-six days, in spite of the degenerated condition of the testis at this period after vasa-efferentia ligation). This comparison demonstrates that the ablation of the pitui-

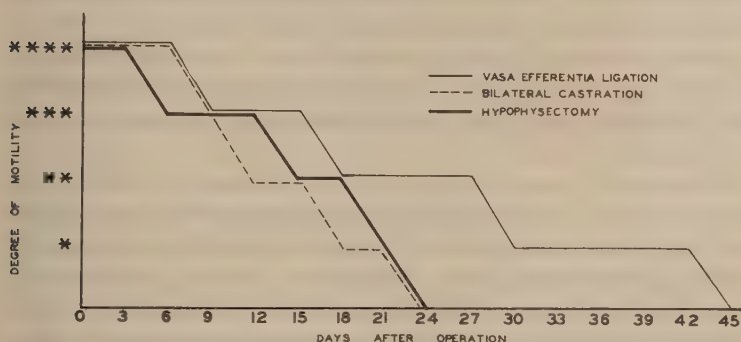


Fig. 2 Figure showing graphically the maximum motility of spermatozoa from the tail of the epididymis for various days after hypophysectomy and after bilateral castration and bilateral vasa-efferentia ligation in non-hypophysectomized rats.

tary has a markedly deleterious effect on the survival of spermatozoa. Furthermore, the effect is as great as after bilateral castration, following which sperm survive for twenty-one days in the tail of the epididymis, for twenty-one days in the ductus deferens, and for nine days in the head of the epididymis. These data demonstrate the profound influence which the pituitary exerts over the mature male germ cell.⁸ Figure 2 shows graphically the maximum degree of

⁸ It is realized that absolute survival periods could be suggested only by a statistical treatment of a large number of individuals, and also the personal equation is a definite factor in an investigation of this kind. For these reasons, we have considered three-day intervals to be a minimum significant survival time. In one animal there was some doubt as to whether the pituitary ablation was complete. This animal was killed on the twenty-third day to make room for other experiments. Slight motion was found and no hypophysis was present at autopsy, it was not included in the tables.

motility of sperm from the tail of the epididymis at various days after each of these operations.

Hypophysectomy and bilateral vasa-efferentia ligation

Mitotic figures are present in sections of testes removed from hypophysectomized animals several days after operation. It seemed possible, therefore, that the testis for a time continued to add sperm to those already present in the epididymis and that without this addition the survival time in hypophysectomized animals would have been even shorter. To check this possibility, a double operation, hypophysectomy and bilateral vasa-efferentia ligation, was performed on additional rats.

The animals were examined from fifteen to twenty-four days after the two operations which were performed within half an hour of each other. The results, given in section B, table 2, demonstrate that spermatozoa survive in hypophysectomized animals, whether vasa-efferentia ligated or not, for twenty-one days. If there is a short postoperative formation of sperm in the hypophysectomized rat, it continues for too short a period to be evident in our observations employing examinations at three-day intervals.

There are other possible interpretations of these results. In view of the fact that mitotic figures are seen in the testis for a few days after hypophysectomy, but sperm survival is very little if any longer in these animals with testes intact than in others with ligated vasa efferentia, it is possible that spermatozoa are formed in the testis for a few days after hypophysectomy, but are not conveyed from the seminiferous tubules into the rete testis and thence into the epididymis, due to the incapacitation caused by the removal of the gonadotropic pituitary hormone. Another possibility is that this pituitary hormone is still present in sufficient quantities after hypophysectomy⁹ to cause morphologically mature sperm to

⁹ In a recent paper (Smith and White, '31) it has been demonstrated that apparently normal corpus-luteum formation continues for about two days after hypophysectomy in the rabbit. This would suggest that the pituitary hormones may be present for some little time after total ablation of the gland.

be formed for several days, but that the endocrine function is more immediately affected so that these sperm are unable to survive beyond a minimum time.

Hypophysectomy and bilateral castration

The fact that the testes atrophy after pituitary ablation and that the sperm-survival period in the hypophysectomized and in the castrated animals is approximately the same suggested that the shortened period of sperm survival after the two types of operations is due to the same factor—the loss of the secretion of the testis. Nevertheless, the possibility remained that the testis supplied an internal secretion after hypophysectomy and also that the pituitary secretion directly contributes to the survival of the sperm in animals which were castrated only. These possibilities could be tested by an examination of animals which were both hypophysectomized and castrated, for if the pituitary secretion has any direct effect on sperm survival or if the testis continues to liberate an internal secretion for a significant period after hypophysectomy, then the sperm after these double operations should survive for a shorter period than after either operation alone. The data (table 2, section C) clearly demonstrate that this is not the case. Sperm live in castrated rats for about twenty-one days regardless of whether the pituitary is present or not. After hypophysectomy the testes do not continue, for any period determinable by the procedure followed here, to secrete a hormone which contributes to the survival of sperm nor does the pituitary liberate a secretion which directly affects their survival in the epididymis.

DISCUSSION

Spermatozoon vitality has interested various workers for some time. The survival of sperm after being introduced into the female tract has been investigated by Lewis ('11), Siegel ('16), Anderson ('22), Hammond and Asdell ('26), and Yochem ('29). Tournade ('13), Wolf ('21), and Yochem ('30), among others, have determined the degree of motility

exhibited by sperm removed from the different levels of the male tract. Others have studied the effects of temperature and various reagents on the survival of spermatozoa (Piersol, '93; Young, '27; Moore, '28; Walton, '30; Yochem, '30; and Granzow, '32). Still others have investigated the effects of various procedures—castration, ductus-deferens ligation, elevation of the testis into the abdomen, unilateral castration, x-ray treatment, and testis transplantation (Benoît, '26; Young, '27; Moore, '28; Heller, '29, and many others).

Although it has been shown that the pituitary gland exerts a powerful regulatory influence over the reproductive system,¹⁰ its influence on the duration of life of fully formed male germ cells has not been investigated. The results which have been given in previous sections demonstrate that the pituitary has a very pronounced effect on the survival of the spermatozoa.¹¹ That this effect is not direct, but is entirely mediated through the testis, has been demonstrated by a double operation, hypophysectomy and bilateral castration, after which spermatozoa survive for the same period (twenty-one days) as after hypophysectomy alone.

¹⁰ The literature on the pituitary-gonad relationship is extensive and will not be reviewed in this paper. Reviews of the subject can be found in the papers of Smith and Engle ('27) and Smith ('30), among others.

¹¹ We have used motility as our criterion for survival in these experiments. Fertility is lost some time before motility ceases (Cohn, '18), and even very active sperm may lack fertility due to genetic constitution or other causes. We realize that fertility itself is perhaps of more interest to animal breeders than the absolute survival times of spermatozoa, but we were interested in employing some criterion of survival which would have as few variables as possible and which could be used from day to day without involving unknowns or, at least, variables, such as the period of the female cycle in which the sperm were inseminated (hypophysectomized animals lose all desire for coitus), the reaction of the vaginal secretions, variations in the ovulation time, the discrepancies due to the sperm remaining outside of the animal for different intervals, the results of using different densities of sperm suspensions for insemination (Walton, '27), and other factors. Motility is the *sine qua non* of fertility in mammalian spermatozoa. It is readily recorded in different degrees and gives easily accessible data for the relative and ultimate survival of sperm. For these reasons we consider motility to be the most definitely comparable criterion of spermatozoon survival for investigations of this kind.

That the epididymis, through its secretions, is directly responsible for the survival of spermatozoa beyond a minimum time was indicated by the research of several workers, notably Van der Stricht ('93), Redenz ('24), and especially by that of Benoît ('26) who, from a series of bilateral and unilateral castrations, drew the conclusions that: spermatozoa held in the epididymis and ductus deferens conserve their vitality and acquire their motility through the secretory products of these organs and that bilateral castration entails, with the arrest of the secretion, the death of the sperm; unilateral castration, on the contrary, which does not affect the glandular activity of the sperm passages leaves the spermatozoa unharmed on the operated side. This differential time of survival of sperm has been confirmed and extended in a series of experiments by Moore ('28). Although Hammond and Asdell ('26) and Young ('31) doubt that a specific secretion of the epididymis is involved and consider that the changes which the sperm undergo in the epididymis are a simple ripening process which could possibly continue under a different environment, nevertheless, it is unquestionable that sperm survive much longer in animals with testes present than in castrated individuals, either through a direct action of the testis hormone on the sperm or indirectly through its action on epididymis.

After hypophysectomy, the epididymis, as is the case of the other accessories, undergoes a profound atrophy. This fact together with the experimental analysis given earlier in the paper indicates that the immediate cause of the shortened period of sperm survival is the same after hypophysectomy as after castration, namely, an atrophy and secretory failure of the epididymis.

The experiments reported here do not supply any conclusive evidence upon which tissue of the testes secretes the male sex hormone. Both the seminiferous elements and the interstitial cells decrease in amount, although the latter seem to be the less profoundly affected. Although they are less affected than the tubules structurally, any secretory activity which they have may be profoundly decreased.

From the foregoing discussion it is evident that the total ablation of the pituitary causes a complete and almost immediate cessation of testicular functioning. A favorable temperature and an adequate vascularization have been considered the chief factors necessary for a testis to cause maximum survival of spermatozoa. However, a properly functioning pituitary is also a major factor and is just as essential for the survival of spermatozoa as it is for their formation.

I wish to take this opportunity to express my appreciation to Dr. Philip E. Smith for the advice and assistance which he has given during the course of this work and in the preparation of the manuscript.

SUMMARY AND CONCLUSIONS

Sperm-motility determinations have been made in three series of hypophysectomized rats. The first series was hypophysectomized only. In addition to hypophysectomy, the second series was vasa-efferentia ligated, and the third bilaterally castrated. The readings were compared with non-hypophysectomized rats of the same colony, one series of which was bilaterally castrated, the second unilaterally castrated, and the third vasa-efferentia ligated. The determinations were made from the head of the epididymis, the tail of the epididymis, the ductus deferens, and in the non-castrated animals from the testes also.

Motility was found to persist in the hypophysectomized rats in each of these regions of the reproductive passages for the same time as in the corresponding regions of the non-hypophysectomized castrated animals. The readings from the tail of the epididymis were the most uniform, the capacity to exhibit motility persisting there for twenty-one days in each type. This is much shorter than after unilateral castration only (forty-five days) or vasa-efferentia ligation only (forty-two days).

Hypophysectomy thus has a pronounced deleterious effect on the survival of sperm in the male reproductive tract.

Vasa-efferentia ligation does not decrease the survival time of epididymal sperm of hypophysectomized animals, indicating that there is no postoperative contribution of sperm from the testes.

Castration also does not decrease the survival time of the sperm in hypophysectomized animals, demonstrating that no internal secretion beneficial to sperm survival is elaborated by the testes after the removal of the pituitary.

The pituitary does not supply any substance which is directly beneficial to sperm survival as demonstrated by the fact that the period of survival is no greater in castrated non-hypophysectomized rats than it is in the hypophysectomized individuals (twenty-one days in each).

Bilateral vasa-efferentia ligation has been introduced as a technique which allows in each animal two epididymal sperm-motility examinations at different times without deprivation of the testicular hormone, thus giving a maximal survival.

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LIVING RAT EGGS

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ONE PLATE (THIRTEEN FIGURES)

A recent survey by Hartman ('29) indicates the paucity of data giving the definitive sizes of living mammalian ova. So far as we know, only two observations have been made on the living eggs of the rat: one by Bischoff ('44) who examined the eggs of one rat at a stage not definitely known, using a unit of measurement open to doubt (Hartman, '29), and giving a broad estimate (111μ in diameter, including the zona pellucida) in any case; the other by Kirkman and Burr ('13), who based their measurements on two one-cell, three two-cell, and two probably unfertilized eggs. Kirkham and Burr state that the diameter of the rat egg is 0.079 mm., but do not indicate how this figure is arrived at, i.e., whether by averaging data for all seven eggs or for the one-cell eggs alone. A number of measurements have been made, or are deducible from figures of fixed eggs (e.g., Sobotta and Burckhard, '10; Kirkham and Burr, '13; Huber, '15; Kremer, '24; Parkes, '31), but in these cases no accurate estimate of shrinkage is available.

We were interested, therefore, to establish with some exactitude the size of rat eggs, especially during the early stages of development. Our observations allow us also to describe the sequence of events occurring at and after fertilization.

I

We shall describe our general technique in some detail, because it indicates with what relative ease living tubal ova may be obtained for experimental purposes.

Animals were mated late in the afternoon or evening. Usually it took fifteen minutes to an hour (after a given female was found to be on heat) before a successful copulation took place. After the female was placed with the male, vaginal smears were taken every fifteen minutes, and examined for sperm. Successful copulation was timed, therefore, to within fifteen minutes, although for all except animals killed within ten hours after copulation the nearest hour was taken as being sufficiently accurate. The fact that several copulations were necessary before one was successful proved useful when unfertilized eggs were desired. Hartman and Ball ('30) state that "the rat copulates several times (five to twenty) before actually ejaculating." Since it could be determined at the first copulation whether or not the female was on heat, sterile matings could easily be secured without vasectomy. In such cases the females were removed from the males after the first copulation. None of the nine females handled in this way showed any sperm in vaginal smears, nor did the eggs from any of them show signs of being fertilized.

Immediately after the female was killed, the fallopian tube on one side was removed, placed in Ringer's solution at room temperature under a binocular dissecting microscope, and cut into several small sections. Each section was squeezed with two pairs of fine forceps, and amid the debris ejected the eggs were found. Undoubtedly, some eggs were lost in this procedure, but with practice a good number were recoverable, and it afforded a rapid and practical approach to the small, tightly coiled tubes. The second tube was examined from twenty minutes to two hours later.

Eggs were removed for observation onto slides with vaselin-sealed cover slips; attempts at fertilization in vitro were in several cases carried on under paraffin-sealed cover slips. Unfertilized eggs were always surrounded by an adhesive mass of follicle cells (fig. 1), which was removed in part by gentle pressure on the cover-slip. Unfortunately, such treatment, used in our examination of the first twenty eggs observed, resulted at times in the slight flattening of the eggs

(e.g., fig. 2) so that our measurements were probably too large and were omitted in the final tabulation. Because of this obscuring mass of adhering cells, our measurements on unfertilized eggs are less accurate than those made on fertilized eggs which floated free under the cover slip. Follicular eggs were obtained by pricking ripe follicles and floating the ejected eggs in Ringer's solution.

Measurements were made with an ordinary ocular micrometer, using a magnification of $440\times$, thus insuring measurements accurate to about 1μ . A number of eggs were photographed, and calculations of the size taken from the photographs checked satisfactorily with measurements of the same eggs taken directly with the ocular micrometer. Photographing any large number of eggs was found difficult, particularly with cleaved eggs, as these tended to fragment rather soon (fig. 8). Uncleaved eggs, especially those taken before and shortly after fertilization, could be kept for considerable lengths of time without showing changes.

Sperm for fertilizations *in vitro* were obtained directly from the vas deferens of the male. Semen obtained by using the electric-ejaculation method (Moore and Gallagher, '30) was usually rather poor.

II

In table 1 are given our collected data on the time relations of developing eggs taken from fertile matings.

It is rather interesting to note that in a spontaneously ovulating animal like the rat we were unable to find ova in animals killed before eight and one-half hours after copulation, but since only three animals were killed sooner than eight and one-half hours after copulation, no general conclusions are made. Presumably, however, fertilization must occur shortly after ovulation, for we never obtained a complete set of unfertilized eggs in fertile matings. According to Hartman and Ball ('30), rat sperm reach the upper end of the uterus in one hundred seconds, so that even if their passage through the tubes is much slower they are probably

present at or near the tops of the tubes when the eggs are shed.

The sequence of events involved in sperm penetration seems to be much the same as that described for the rabbit (Pincus, '30; Pincus and Enzmann, '32). The sperm pass between the surrounding follicle cells, which float free as the sperm come through, and enter the egg. The freeing of adherent follicle cells is an inevitable accompaniment of sperm penetration (see section on in-vitro inseminations), for all ova

TABLE 1
Ages, numbers, and stages of eggs found after fertile matings

HOURLS AFTER COPULATION	NUMBER AND STAGES OF EGGS FOUND	HOURLS AFTER COPULATION	NUMBER AND STAGES OF EGGS FOUND
0	None	25	7 one-cell
4½	None	27	2 one-cell
6½	None	42	12 two-cell (two animals)
8½	5 one-cell	44	8 two-cell
11	2 one-cell, 10 one-cell or unfertilized (two animals)	49	4 two-cell
12	12 one-cell (two animals)	58	6 two-cell
14	1 one-cell, 2 unfertilized	60	3 two-cell, 1 three-cell
15	5 one-cell	61	4 two-cell, 2 six(?) -cell
16	5 one-cell	67	3 three-cell, 2 four-cell
19	9 one-cell, 1 unfertilized	70	1 three-cell, 2 four-cell
20	8 one-cell	71	1 four-cell, 2 seven(?) -cell
21	5 one-cell	85	1 three-cell, 2 seven(?) -cell 3 eight-cell
22	5 one-cell	94	6 eight(?) -cell
24	6 one-cell	95	3 eight(?) -cell

removed from eleven to twenty-three hours after sterile matings were surrounded by these cells (compare Pincus, '30). As in the rabbit, more than one sperm may pass through the zona pellucida (fig. 3), but presumably the supernumerary spermatozoa remain in the perivitelline space. We have observed them moving in this space, but have never actually seen entry into the egg proper. The fact that rat sperm are longer—about 177 μ in length—than the diameter of the egg should make easy any observation of the entry of the fertilizing spermatozoon. We deduce, therefore, that the fertilizing spermatozoon must enter the egg very rapidly indeed,

and must merge with the egg protoplasm equally rapidly. In only one doubtful case have we observed any identifiable portion of a spermatozoon inside the vitellus proper, and yet we have undoubtedly examined a number of ova very shortly after sperm entry.

We have had some difficulty in using the number of polar bodies present as criteria of the occurrence of fertilization. Of fifty-six one-cell eggs presumably fertilized *in vivo*, in only thirty-six were polar bodies actually seen. Two polar bodies were present in eighteen eggs, one polar body in sixteen, and two eggs showed three polar bodies. While it is probable that polar bodies were overlooked in certain instances, it is notable that Sobotta and Burckhard ('10) never found evidence that the first polar body, formed in the follicle, lasted after ovulation. It seems, therefore, that the criteria employed by Yamane ('30) for rabbit eggs may not apply to rat eggs.

In certain eggs evidently taken very shortly after sperm entry, certain obvious protrusions were observed, resembling very closely the protrusions that precede polar-body formation (e.g., Sobotta and Burckhard, '10; Long, '12). In no case where close examination was made did these protrusions actually form polar bodies (fig. 5). It seems likely that these protrusions represent attempts at polar-body formation which either the low temperature or our method of handling suppressed (Pincus, '30, for instances of cleavage reversals in rabbit eggs).

Our data on the rate of cleavage *in vivo* check (except for ninety-six-hour eggs) those of Huber ('15) made on fixed material (figs. 6 to 9). It is interesting to note that the rate of cleavage is much less than that of rabbit eggs which are larger eggs (Gregory, '30; Lewis and Gregory, '29).

One-cell eggs about to cleave are easily distinguished, because they become somewhat elongated before segmentation. This phenomenon complicated to some extent our measurements. A similar elongation preceding division probably occurs also in the cells of segmented eggs.

III

The more or less spheroidal condition of eggs from the end of the one-cell stage on leads to the construction of the data of table 2 in two sets of measurements, one representing an average of all the shortest diameters, and the other an average of all the longest diameters. Where data on round eggs are included, the same diameter was used for both the long and the short averages. The measurements given for three- to eight-cell stages are the longest diameter of all and the longest diameter at right angles to it; for the two-cell stage, the shorter diameter given is the average of the cross

TABLE 2

Average sizes of rat eggs in several stages of development. All measurements in microns, with probable errors

STAGE	AVERAGE SIZE OF VITELLUS	NUMBER OF MEASURE- MENTS	AVERAGE SIZE OF ZONA PELLUCIDA	NUMBER OF MEASURE- MENTS
Follicular	$85.3 \pm 0.8 \times 88.3 \pm 2.5$	3		
Tubal, un- fertilized	$75.8 \pm 1.9 \times 77.1 \pm 1.7$	16		
1-cell	$70.9 \pm 1.8 \times 76.4 \pm 2.4$	55	$100.5 \pm 2.6 \times 106.5 \pm 2.2$	24
2-cell	$60.7 \pm 2.9 \times 88.0 \pm 1.9$	31	$98.7 \pm 2.3 \times 107.2 \pm 2.2$	23
3- to 8-cell	$68.8 \pm 7.1 \times 90.1 \pm 4.5$	19	$95.3 \pm 4.3 \times 110.8 \pm 3.3$	23

diameters of the two cells. The data for eggs beyond the one-cell stage are the least accurate, because of irregular cell outlines and the varied groupings of individual blastomeres.

The indications from these data are that among one-cell eggs the follicular eggs are much the largest of all, the unfertilized eggs next, and fertilized eggs the smallest. Disregarding the data on follicular eggs, because of the small number examined, it seems obvious that there is a shrinkage of the egg proper at or after fertilization.¹ This accords well with direct observation (figs. 3 and 4), for in fertilized ova the perivitelline space appears larger (compare Pincus and Enzmann, '32).

¹ This shrinkage may be direct or due to the extrusion of cytoplasmic material. Shrinkage has been observed in eggs where no polar bodies were present.

Since the comparisons of diameters offers at best a crude comparison of this shrinkage, we have calculated the volumes of the eggs, round eggs being taken as spheres and elongated eggs as prolate spheroids, that is, solids formed by the rotations of ellipses about their major axes. The formula for the volumes of such solids is: $v = 4/3\pi ab^2$, where a is the major semi-axis, and b the minor semi-axis. These calculations are collected in table 3.

Again we deduce that fertilized eggs show a definite shrinkage when compared with unfertilized eggs, in this case some 14 per cent. Furthermore, since the volumes of elongated eggs check so well with those of round eggs, it is deducible

TABLE 3
Estimated volume of single-celled eggs

STAGE	AVERAGE VOLUME OF ROUND EGGS, CU.MM.	AVERAGE VOLUME OF ELONGATED EGGS, CU.MM.	AVERAGE VOLUME OF ALL EGGS, CU.MM.
Follicular	0.000333(1)	0.000339 $\pm$ 0.000017	0.000337 $\pm$ 0.000010
Tubal, un-			
fertilized	0.000251 $\pm$ 0.000023	0.000226 $\pm$ 0.000013	0.000234 $\pm$ 0.000018
1-cell	0.000202 $\pm$ 0.000009	0.000200 $\pm$ 0.000010	0.000201 $\pm$ 0.000010

that no marked change in volume occurs in one-cell eggs after the initial shrinkage, despite the precleavage activity which is probably the usual cause of elongation. In fact, the available data indicate that the volume of the egg undergoes no change during the cleavage stages observed. Similarly, the size of the zona pellucida remains practically constant throughout.

The initial segmentation results in two slightly unequal cells as in other mammals. This is demonstrated by averaging the data for all the smaller cells together and contrasting it with the averaged data for the larger cells in the two-cell stage. The dimensions of the smaller cells are $42.3 \times 59.2 \mu$, the larger, $45.8 \times 62.5 \mu$.

IV

A final check on our observations was made with a set of attempts at fertilization *in vitro*. Similar experiments performed by Long ('12) resulted in the dehiscence of the follicle cells in a few minutes, initiation of polar-body formation in five minutes to two hours after insemination, and the completion of polar-body extrusion in some forty-five minutes. The eggs observed by Long were kept in Ringer's solution, and never cleaved, but began to degenerate after some twelve hours in a thermostat kept at body temperature. He never saw sperm entering the eggs.

No attempt was made to culture the eggs used in these experiments. Active, and often rather heavy, sperm suspensions were placed with eggs obtained from sterile matings, and the slides containing them, as well as control slides, were placed in incubators kept at 37°C. Slides used for direct observation were kept on a warm stage.

Eggs placed with active sperm were quickly denuded of adherent follicle cells, and a clearly marked perivitelline space was observed (figs. 10 to 12). No actual sperm entry was seen, nor were any spermatozoa seen in the perivitelline space. Neither was polar-body formation observed in eggs examined closely for this phenomenon. A number of eggs, however, exhibited the protrusions previously described for eggs fertilized *in vivo*. Whether these protrusions represent attempts at polar body formation or fertilization cones cannot be said (figs. 10 and 11).

Eggs placed in a suspension of dead sperm (the sperm were killed by heating well below the boiling point) showed also a shrinkage of the vitellus; this shrinkage was less than that observed with eggs placed with living sperm—12 per cent compared with 17 per cent. Furthermore, the dehiscence of follicle cells was not as marked as in preparations containing living sperm (fig. 13). These results may be taken to substantiate Yamane's ('30) contention that a substance (proteolytic enzyme) produced by sperm is the responsible factor for activation of the eggs as well as the dehiscence of

the follicle cells. The obvious alternative is that the presence of sperm may result in a hypertonicity of the medium sufficient to cause the results observed.

Control eggs kept in the incubator without sperm never become free of the adherent follicle cells. Nor did any shrinkage of the egg occur in the controls, whereas a definite shrinkage did occur in eggs placed with sperm. The data of table 4 demonstrate this clearly. It will be noted that the shrinkage accompanying insemination must be very rapid indeed, since our second observation of inseminated eggs

TABLE 4

Data on size of unfertilized eggs kept under various conditions in vitro

TREATMENT	NUMBER OF EGGS	AVERAGE DIAMETER IMMEDIATELY AFTER PUTTING EGGS ON SLIDE, MICRONS	AVERAGE VOLUME, CALCULATED, CU.MM.	AVERAGE DIAMETER SOME TIME AFTER INCUBATION, MICRONS	AVERAGE VOLUME, CALCULATED, CU.MM.	SHRINKAGE, PER CENT
Incubated in Ringer's solution alone	7	74.4 $\pm$ 1.4	0.000216	76.3 $\pm$ 0.4	0.000232	—
Incubated with live sperm	9	77.9 $\pm$ 1.4	0.000248	72.7 $\pm$ 1.4	0.000205	17
Incubated with dead sperm	10	72.8 $\pm$ 1.1	0.000204	69.7 $\pm$ 1.3	0.000179	12

often was made only ten minutes after insemination. Furthermore, in one case where two eggs were placed in an extremely heavy sperm suspension definite shrinkage was observable in five minutes after insemination. The fact that our sperm suspensions were probably much heavier than those occurring in the tubes prohibits any deductions concerning the time relations of the events accompanying fertilization in vivo.

SUMMARY

1. Unfertilized rat eggs may usually be obtained without vasectomy by removing the female from the male after the first copulation.

2. Unfertilized eggs may remain in the fallopian tubes surrounded by follicle cells until at least twenty-three hours after copulation. The follicle cells surrounding fertilized eggs are dispersed at fertilization.

3. Ovulation may occur as early as eight and one-half hours after copulation.

4. The first cleavage comes between twenty-seven and forty-two hours after copulation. The second and third cleavages come between sixty and ninety-five hours after copulation.

5. The average size of the fertilized, unsegmented egg is about $71 \times 76 \mu$. There is probably not much change in size through the first three cleavages.

6. The two cells resulting from the first cleavage are slightly unequal in size.

7. At the time of fertilization there is a shrinkage in volume of the egg of about 13 to 17 per cent.

8. Eggs placed in vitro with either dead or living sperm show a shrinkage comparable to that occurring in vivo.

9. There is a rapid dispersion of the follicle cells of eggs placed with live sperm in vitro. This is not so marked when dead sperm are used, and no dispersion at all occurs in incubated eggs without sperm.

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PLATE 1

EXPLANATION OF FIGURES

- 1 Sixteen hours after a sterile mating. Note surrounding follicle cells.
× 184.
- 2 Eleven hours after a fertile mating. One spermatozoon about egg at left.
× 181.
- 3 Eleven hours after a fertile mating. One spermatozoon clearly above the egg; a second outlined at right above and between polar body and first spermatozoon. × 181.
- 4 Eighteen hours after a fertile mating. Note shrunken egg and at least one polar body. × 184.
- 5 Eight and one-half hours after fertile mating. Note protrusion resembling polar-body formation; also spermatozoon above the zona pellucida. × 184.
- 6 Forty-two hours after a fertile mating. Two cells. × 200.
- 7 Sixty hours after a fertile mating. Three cells, three(?) polar bodies.
× 327.
- 8 Seventy-one hours after a fertile mating. Seven(?) cells, one polar body. The egg is beginning to fragment. × 184.
- 9 Eighty-five hours after a fertile mating. Eight cells, one polar body.
× 184.
- 10 Sixteen hours after a sterile mating, with living sperm one-half hour. Note absence of follicle cells and protrusion resembling polar-body formation.
× 184.
- 11 Same egg as in figure 10 after five hours at room temperature. Protrusion subsided. × 184.
- 12 Eleven hours after a sterile mating, with living sperm two hours. Note shrunken egg and two polar bodies. × 154.
- 13 Seventeen hours after a sterile mating, with dead sperm two hours. Note scarcely dispersed follicle cells and polar body. × 184.

